# For Reference

THE UNIVERSITY OF ALBERTA

THE INTERACTION OF <u>STAPHYLOCOCCUS</u> <u>AUREUS</u>

STAPHYLOPHAGE 7 AND ANTIBIOTICS,

<u>IN</u> <u>VITRO</u> AND <u>IN</u> <u>VIVO</u>

by

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The undersigned certify that they have read, and

recommend to the Faculty of Graduate Studies for acceptance,

a thesis entitled THE INTERACTION OF STAPHYLOCOCCUS AUREUS

STAPHYLOPHAGE 7 AND ANTIBIOTICS, IN VITRO AND IN VIVO

submitted by Leslie Athiel Howard in partial fulfilment of

of the requirements for the degree of Master of Science.

ABSTRACT

The growth of <u>Staphylococcus aureus</u> PS7 infected with staphylo-
coccus bacteriophage 7 was examined at 37$^{\circ}$C.  Efficient lysis of
suspensions of this bacterial strain by Ø7 in nutrient broth required
the addition of $CaCl_2$ in concentrations greater than 75 µg/ml.  One-step
growth experiments indicated a burst size of ca. 95.  Absorbance deter-
minations at 600 mµ were used to show the extent to which the turbidity
of log-phase suspensions of <u>S. aureus</u> PS7  was influenced by Ø7 in the
presence of the following antibiotics: penicillin, vancomycin, bacitracin,
D-cycloserine and chloramphenicol.  When added immediately after
penicillin, bacitracin or D-cycloserine, Ø7 was able to effect lysis and
reduce the absorbance of suspensions of <u>S. aureus</u> PS7 to half the initial
value in a shorter period of time than could either Ø7 or antibiotic
alone.  Chloramphenicol produced no such reduction in turbidity during a
comparable period of observation (4 - 5 hours) at 37$^{\circ}$C.  When Ø7 was
added immediately after vancomycin, lysis of <u>S. aureus</u> PS7 was inhibited.
However, if the addition of Ø7 preceded vancomycin by 10 minutes,
efficient lysis occurred and a half-lysis time less than that for either
Ø7 or vancomycin alone was obtained.  Using a Ø:cell ratio of 1:40, the
adsorption rate of Ø7 to <u>S. aureus</u> PS7 was found to be 85%, 95% and ca.
100% complete in 2½, 5 and 10 minutes respectively.  The rate and extent
of adsorption was not affected by the presence of the antibiotics
penicillin, vancomycin, bacitracin, D-cycloserine and chloramphenicol.

Because <u>S. aureus</u> PS7 was not lethal for mice except at doses in

the order of $10^{10}$ cfu, another strain of coagulase-positive staphylo-coccus, S. aureus 19, selected for its lethality on intraperitoneal inoculation in mice ($LD_{50}$ = 2.9 x $10^7$ cfu) and for its ability to act as host for Ø7, was used to determine the extent to which each of the anti-biotics chloramphenicol, vancomycin, penicillin and oxacillin, in combination with Ø7, influenced the survival of infected mice. Statistical analysis of the results did not permit the conclusion that Ø7 and anti-biotics interact to provide additive protection when given immediately after intraperitoneal inoculation of mice with S. aureus 19. Such an inference is however suggested by the data obtained when the infecting dose of S. aureus 19 was ca. 8.8 x $10^8$ cfu, the Ø:cell ratio was ca. 3:1 and the chloramphenicol, vancomycin, penicillin and oxacillin doses were 1000 µg, 200 µg, 1000 µg, and 2000 µg respectively. Here, all the mice receiving Ø7 plus antibiotic showed a greater number of survivors than those receiving Ø7 or antibiotic alone. It seems likely that significant interaction was not demonstrated because the sample unit (10 mice) was too small.

A 15-minute delay in the administration of Ø7 following intra-peritoneal inoculation of mice with S. aureus PS7 resulted in considerable loss of protective action. This loss of protective action appeared to be unaffected by the presence of antibiotics. Mice which were pre-treated with 500 units of antitoxin (Connaught, Canada) prior to infection with S. aureus 19 showed a significant delay in the onset of death.

## ACKNOWLEDGEMENTS

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I would also like to express thanks to the drug distributors listed in Appendix I for the gift of antibiotics used in this investigation.

## LIST OF ABBREVIATIONS AND DEFINITIONS

Amax            – maximum absorbance

Bacit.          – bacitracin

cfu             – colony-forming units

Chloramph.      – chloramphenicol

D-cyclos.       – D-cycloserine

EOP             – efficiency of plating

Oxac.           – oxacillin

Pen.            – penicillin

pfu             – plaque-forming units

RTD             – routine test dilution

$t\frac{1}{2}$          – half-lysis time = time required for absorbance to
                  to return to half its initial value

ti              – time required for absorbance to return to its
                  initial value

Ø               – phage, bacteriophage

Ø:cell          – pfu:cfu

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INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Since the discovery of bacterial viruses by Twort (92) and

d'Herelle (13), early workers including d'Herelle himself directed

their attention to use of bacteriophages in the treatment of bacterial

infections. The early literature abounds with references to attempts

made at the clinical level to cure a variety of bacterial infections,

including those caused by staphylococci, dysentery, and cholera, by

means of bacteriophages. The reported results of much of this early

work are highly equivocal and difficult to evaluate.

The advent of antibiotics and the remarkable success which

these had as chemotherapeutic agents resulted in the virtual abandonment

of work directed towards the investigation of bacterial viruses as anti-

bacterial agents capable of being applied therapeutically. However, the

emergence and spread of antibiotic-resistant bacteria, as well as the

occurrence of many chronic and refractory infections, especially those

due to staphylococci, has prompted some renewal of interest in bacterio-

phages as possible therapeutic adjuncts.

It is generally accepted that clinical application of a new

method of therapy, for example a new antibacterial agent, should be

preceded by thorough laboratory investigations both with respect to its

mechanism of action _in vitro_ as well as to its evaluation _in vivo_ through

the use of experimental animals. The history of the use of bacteriophages

for chemotherapeutic purposes shows that this process of laboratory investigation prior to clinical usage has not been strictly adhered to. Probably because early workers such as Larkum (51) found bacteriophage lysates were innocuous or because of urgent need, extensive clinical use was made of these lysates for therapy before a sufficiently satis-factory explanation for their _in vitro_ and _in vivo_ mode of action had evolved. Even to-day, despite the vast amount of information which has accumulated on bacteriophages and viruses, and the many and sophisticated techniques which have been developed for their investigation, knowledge pertinent to the _in vivo_ interaction of bacteriophages and bacteria cannot, even with much generosity, be described as adequate.

The present study using staphylophage 7 and _Staphylococcus aureus PS7 in vitro_, and _Staphylococcus aureus 19 in vivo_ in mice, was undertaken in an attempt to gain some understanding of the _modus operandi_ of bacteriophages and bacteria in relation to therapy and to examine the influence of some antibiotics in this interaction.

LITERATURE REVIEW

A number of reviews of the early literature on bacteriophage therapy have appeared, the most comprehensive being those of Eaton and Bayne-Jones (16), Krueger and Scribner (45), and Morton and Engley (59).

In this review, two main aspects of the literature will be presented; first the clinical use of bacteriophages will be discussed, and then consideration will be given to those experiments performed in vivo and in vitro which have attempted to provide an acceptable rationale for the use of bacteriophage lysates in the therapy of bacterial infections. In addition, some attention will be given to the study of experimental staphylococcal infections and other topics which provide a suitable background for understanding the interactions involved in the complex systems under investigation.

Bacteriophage in the treatment of staphylococcal infections.

According to Eaton and Bayne-Jones (16) a great number of the favorable results of bacteriophage therapy have come from the use of this agent in staphylococcal infections. MacNeal and Frisbee (55) have reported favorably on the treatment of staphylococcal septicemia with bacteriophage lysates and ascribed the beneficial effect to augmentation of the immune action of body fluids and phagocytosis. Larkum (52) has presented a summary of a study undertaken by the Michigan Department of Health, under whose auspices the polyvalent Gratia strain of staphylo-coccus bacteriophage was distributed for therapeutic use. Favorable results were obtained for the treatment of furunculosis, acne, osteo-

myelitis and miscellaneous other staphylococcal diseases. Surgical in-
fections have been subjected to bacteriophage therapy by Bazy (9) who
recorded effectiveness especially where staphylococci were involved.
Numerous other reports on the use of bacteriophage lysates in the therapy
of staphylococcal infections have been summarized in tabular form by
Larkum (52), and others have been cited in the reviews of Eaton and Bayne-
Jones (16) and of Krueger and Scribner (45), and will not be repeated here.

Despite some favorable claims, the history of the use of staphy-
lococcus bacteriophage in the treatment of disease has recorded many
failures, especially where urinary tract infections were involved, as
the publication of Krueger, Faber and Schultz (44) shows. A number of
these unfavorable reports have been referred to by Elek (17), whose
comments reveal a decided lack of enthusiasm for this approach to the
treatment of bacterial infections.

Bacteriophage in the treatment of enteric diseases.

The literature on the use of bacteriophages for therapy of enteric
diseases is voluminous and difficult to evaluate. The comprehensive
review of Morton and Engley (59) would seem to suggest that the results
here were even more equivocal than with staphylococcal infections. These
authors have drawn attention to the reports most frequently cited either
for or against the use of bacteriophages in the treatment of enteric
infections. They have criticized these as being in general
"unscientific" and have pointed out that in most instances proper controls
had not been included and that the data were inadequate for statistical
evaluation.

## Bacteriophage in the treatment of cholera.

As with staphylococcal and enteric diseases, both favorable and unfavorable reports have appeared on the use of cholera bacteriophages in the treatment of clinical diseases. Dutta and Panse (15) have cited some of the results which have appeared between 1926 and 1945. Shortly after this period the use of bacteriophages for the treatment of cholera was virtually abandoned.

Recently, however, results of a team of Russian workers in East Parkistan and Afghanistan have been reported by Sayamov (76). In this study cholera phage prepared by cultivation on cholera vibrios by alternate passage in the small intestine of guinea pigs and in bile was found to have therapeutic and prophylactic value. Significant improvement was obtained in the majority of over 150 patients treated, and, in seven locations where phage was given prophylactically to over 30,000 people no case of cholera requiring hospitalization occurred. The effectiveness of the Russian materials and methods was later confirmed by Mukerjee (61).

## Bacteriophage in animal experiments and in the treatment of experimental staphylococcal infections in mice.

Although for the most part thorough investigations of the suitability of bacteriophages for therapy in laboratory animals have been relatively few, especially when the extensive clinical use which has been made of lysates is considered, some pertinent studies have neverthless been recorded.

Arnold and Weiss (4) obtained some protective effect by simultaneous injection of bacteriophages and organisms of the typhoid-dysentery

group into mice and rabbits. They showed that a 30-minute delay in the administration of phage was sufficient to abolish its positive influence on the course of the infection. The Rakietens (67) found that developing chick embryos could be protected from death caused by Flexner's bacillus by administration of bacteriophage in the chorioallantois as late as five hours after infection of the embryos. In addition they obtained a correlation between the survival of the embryos and demonstrable proliferation of bacteriophage.

Walker (97) studied the effect of bacteriophage on the size, appearance, and character of lesions produced by intradermal injection of staphylococci into shaven rabbits, and compared the responses with those due to the influence of chemical disinfectants such as phenol and mercuric chloride. He concluded that phage was effective in reducing the size of the lesions produced, over a wider range of dilutions and moreover it was less toxic than the chemicals studied.

The recent revival of interest in bacteriophages as therapeutic agents has prompted investigations using mice infected with staphylococci. Muir and Blakemore (60) studied the effect of staphylophage 81 on the mortality of mice infected intraperitoneally with S. aureus strain 80/81. Using an infecting dose of $1 - 2 \times 10^9$ cfu ($20 - 40 \times LD_{50}$) they observed that mice died in $5 - 6$ hours post-infection and that mortality was close to 100%. By treating immediately with staphylophage 81 in a phage:cell ratio of 1:1 they were able to achieve 72% survival, and to show that the degree of protection was related to phage dose.

Bartell et al. (7) with the same staphylococcal and staphylophage

strains essentially confirmed the observations of Muir and Blakemore (60). However, they went on further to demonstrate in vivo interaction of phage and bacteria, as evidenced by the higher and persisting titers of circulating phage in mice infected with S. aureus 80/81 as compared with uninfected or heterologously infected animals. A later study by Bartell et al. (8) confirmed in vivo interaction of bacteria and phage and in addition showed an adsorption period reflected in a drop in the initial levels of circulating phage, with subsequent significant rise of the titre. They obtained maximal protection at a phage:cell ratio of 1:2 and proved that the protective action of the staphylophage lysates was dependent on the presence of active phage particles. Bartell et al. (7) also showed that whereas phage given up to 3 hours before bacteria was still able to protect mice, a reversal of the order of intraperitoneal injection resulted in a rapid decrease in protection, falling to no protection if phage was delayed for half an hour. They also obtained a relation between protective ability and the level of circulatory phage; e.g. when phage concentration was ca. $10^5$ pfu/ml protection was good, but it was poor, or absent, at levels less than $10^3$ pfu/ml.

Treatment of infections with bacteriophage in combination with other agents.

In many of the studies where bacteriophage had been employed therapeutically, this agent had not been used alone, rather, in addition to standard methods of treatment. This limitation of much of the published work on phage therapy has been pointed out by Elek (17).

The report of MacNeal, Filak and Blevins (54) is the one most most often cited as a study directed specifically to the evaluation of

another agent in conjunction with bacteriophage. These authors claim

that penicillin and staphylophage are effective in the treatment of

osteomyelitis, facial carbuncle and endocarditis. They also report

success for the treatment of endocarditis due to streptococci moderately

resistant to penicillin, when a combination of penicillin and strepto-

coccus bacteriophage was used.

The immunological approach to bacteriophage treatment.

The observations of d'Herelle in his study of haemorrhagic

septicemia in water buffaloes indicated that bacteriophage filtrates were

capable of producing immunity. Larkum (51) for example claims that phage

lysates were better antigens than the bacterial suspensions themselves.

Arnold and Weiss (3) have placed emphasis on the immunological aspects of

phage therapy and have demonstrated opsonic, precipitating, and

complement-fixing antibodies to bacterial proteins in immune sera

prepared from phage lysates.

Immunological mechanisms, both specific and non-specific, have

been invoked in order to provide an explanation for the therapeutic action

of bacteriophage lysates. It is not surprising therefore that these

phage lysates have been used specifically for immunological purposes.

Larkum (51) reports on the use of bacteriophage lysates as a

substitute for typhoid vaccines. Mention has already been made of the

successful use by Sayamov and Mukergee (61) of cholera bacteriophage for

prophylaxis.

More recent use of bacteriophage lysates in provoking immunity

mechanisms was made by Baker (4) who prepared lysates from the wide

spectrum Gratia strain of staphylophage and used them with success,
topically and parenterally, for the treatment of allergic and chronically
infected patients, and for those with antibiotic-resistant staphylococci.
Wittig, Raffetto and Bason (100) conducted a controlled double blind
study but were unable to find any significant advantage from the use of
Baker's lysate (4) in the treatment of so-called infective asthma in
children.

In vitro studies with bacteria, bacteriophage and antibiotics.

Himmelweit (31) proposed that a combination of bacteriophage and
penicillin might produce an enhanced effect on susceptible bacteria
provided the action of the bacteriophage was not interfered with by the
penicillin. Using Staphylococcus aureus S3K he demonstrated that the
effect of a combination of penicillin and staphylophage on this organism
was at least additive. He also indicated that the difference in the
rates of lysis for phage used alone, and phage used in combination with
penicillin was more pronounced in logarithmic phase cultures. The
conclusions arrived at by Himmelweit (31) were later confirmed by
Nicolle and Fauget (64).

A more extensive study of the interaction of Staphylococcus
aureus, staphylophage and penicillin was conducted by Krueger et al.
(43). Working with the K strain of Staphylococcus aureus and staphylo-
phage K, these workers concluded that the time required for the initiation
of massive lysis of the cocci by phage was considerably reduced in the
presence of penicillin and that the accelerating action was independent

of the concentration of penicillin over a wide range (0.1 - 1 x $10^4$

units/ml). Elford (18) essentially confirmed these observations using

the same strains of organism and phage. He stated, in addition that

concentrations of penicillin far in excess of those inhibitory to growth

and acting for longer than the generation time, did not influence the

adsorption of phage, and deduced from this that bacteriophage receptor

sites were not affected by the antibiotic. Although the normal rate of

phage multiplication was reduced, rapid lysis occurred nevertheless, and

by control experiments with phage-free ultra-filtrates and with heat-

inactivated phage, he confirmed that the lytic phenomenon was dependent

on active phage particles. As an explanation of his observations, Elford

(18) made the following proposals:

(1)     Penicillin inhibits the growth of the organisms initially not

infected with phage so that at each stage lysis in the presence of

penicillin produces a proportionately greater reduction in turbidity.

(2)     Cells whose growth has been affected through the action of

penicillin, while they no longer support the normal multiplication of

phage, are nevertheless equally susceptible to lysis.

(3)     The lytic potencies of penicillin and phage are supplementary.

Experimental staphylococcal infection in mice.

Many studies of experimental staphylococcal infection in animals

have been conducted. Smith (83) for example, investigated the effect of

intravenously administered S. aureus in 3 - 4 week-old mice and found that

the organisms were plentiful in the spleen, but declined over a period of

two weeks.  The number of staphylococci in the lungs was found to be
initially lower than that in the spleen, although the organisms
persisted longer in the lungs.  In the kidney the organisms were present
at first in small numbers but later there was multiplication with an
increase in the population.  In addition, Smith (83) showed a correlation
between fatality and kidney malfunction as revealed by histological
studies and creatinine determinations.  McCune, Dineen and Batten (57)
also found that high population levels in the kidneys were associated
with abscesses and destruction of renal tissue.

The histopathological investigations of Gray et al.(29) confirmed
the involvement of the kidneys and showed focal necrosis and mild random
interstitial infiltration of polymorphonuclear neutrophils in both
cortical and medullary regions with late secondary necrosis of the
pelvis of the kidney, and urinary bladder; other organs appeared to be
less affected.  The liver showed many micro-necrotic foci and some peri-
vascular polymorphonuclear infiltration.  The spleen and renal lymph
nodes showed hyperplasia of reticulum cells, at first mild but more marked
by 10 days post-infection.  These histopathological observations of staphy-
lococcal infection in mice are similar to those of Lovel and Cotchi (53)
using Corynebacterium renale II; the essential difference arising from
use of the two organisms was that whereas with C. renale II the papillary
region of the kidney was primarily involved, staphylococcal infection
showed in addition abscesses in the cortical and medullary zones.

Gorrill (27) and Smith and Dubos (84) showed the importance of
inoculum size in producing experimental infection of staphylococci in

mice.  The investigation of Gray et al. (29) suggests that kidney

infection is due to the growth of a relatively small percentage of the

original inoculum, less than $10^5$ cfu being unable to get past the

combined phagocytic potential of the neutrophils and reticuloendothelial

cells.  Sellers and Le Maistre (81) showed that there was a critical

number of staphylococci (greater than $10^5$ cfu) required intravenously to

produce characteristic kidney population curves.  The generally accepted

view that the fixed macrophages of the liver and spleen play an important

part in the removal of intravenously inoculated bacteria was confirmed

by Gray et al. (29), and counts suggested these organs were the most

efficient in phagocytosis.

The studies of Smith et al. (85) demonstrated the intraperitoneal

route was among the most lethal for mice infected with staphylococci.

However, with high inocula up to $10^{11}$ cfu, these workers were unable to

reduce the interval between inoculation and death to less than 60 minutes.

They showed that death resulted from live cocci but were unable to define

a particular target organ as had been suggested by previous workers.

The influence of antibiotics on experimental staphylococcal infection.

Sellers and Le Maistre (81) showed marked antibacterial effect,

as a result of 1.5 mg daily dose of penicillin, on S. aureus Sa 235 in

mice.  There was a significant decrease in the bacterial population of

the kidney, although a similar decrease in the spleen, greater than that

for the control, did not occur.  Sellers and Le Maistre (81) also showed

that cortisone caused an increase in bacterial population greater than

occurred in the control and that the spleen lost its ability to effect a

decline in the bacterial census, high levels being maintained. McCune,

Dineen and Batten (57), using the penicillinase-producing Giorgio strain

of Staphylococcus aureus in mice, studied the effect of penicillin,

streptomycin and novobiocin on the bacterial populations in the spleen,

kidney and lungs. Penicillin alone caused a slight decrease in the

bacteria in the kidneys and promoted increased survival of the animals.

A more marked effect was obtained with streptomycin. Penicillin and

streptomycin together were less effective than streptomycin alone. A

similar series of observations was obtained for the bacterial populations

in the lung; penicillin showing slight effect, streptomycin showing

increased effectiveness, while penicillin plus streptomycin were less

effective than streptomycin alone. In the spleen there was also a low

order of influence with penicillin; streptomycin alone or in combination

with penicillin was able to bring about a marked reduction in the

bacterial census of this organ.

An important observation of Sellers and Le Maistre (81) was that

despite the effectiveness shown by the drugs singly or in combination,

staphylococci persisted in the spleen, kidney and lungs of the majority

of the animals even after 28 days of therapy. This continued presence

of organisms could not be explained by the emergence of drug resistance

in the usual sense.

Failure to eliminate some infectious processes by antibiotic

therapy has led to a number of investigations of the fate of intra-

cellular bacteria in the presence of environments containing chemothera-

peutic drugs. Baker (5), using mononuclear phagocytes from the pleural

cavity of albino rats in tissue culture, studied the fate of a strain of

Staphylococcus aureus when penicillin and streptomycin were present.

He concluded that multiplication and death may be occurring simultaneously

in populations of intracellular organisms.  He also reported that both

penicillin and streptomycin showed reduced activity on intracellular

organisms.  In addition, with the suppression  of extracellular growth by

the antibiotics, continued phagocytosis resulting in marked increase in

the intracellular population of organisms was abolished.  Rogers and

Tompsett (71) reported multiplication of organisms intracellularly.  This

view has been challenged by Kapral (35) who presented data to show that

virulent staphylococci survived but did not multiply within normal human

and rabbit leucocytes, and were actually destroyed by rat leucocytes.

Kapral's work however supports the assertion that when an increase in the

extracellular population was prevented by antibiotics, continued phago-

cytosis with increase in the intracellular census, did not occur.  Using

a continuous flow technique he showed that this limitation of the intra-

cellular population of cocci was not the result of penetration and action

of the antibiotics where these were employed.  A reasonable explanation

for the mechanism of intracellular killing of bacteria in neutrophils has

been presented by Hirsch and co-workers (32), who have demonstrated the

discharge of lysosomal contents into phagocytic vaculoles.

Binding and distribution of antibiotics in vivo and in vitro.

According to Rolinson (73) "the element of uncertainty regarding

the exact role of protein binding in chemotherapy is encountered widely

in the published work, both in reviews and in original papers".

Similar uncertainty in regard to the significance of serum levels of

antibiotics to therapy has been expressed.

In general, the combination of antibiotics with serum proteins

is reversible, as pointed out by Rolinson and Sutherland (74) and Klotz,

Urquhart and Wendell (40). This dynamic state is expressed by Rolinson

(72) as follows:"Protein + free antibiotic $\rightleftharpoons$ protein-bound

antibiotic". The more important factors influencing the amount of anti-

biotic bound at any moment are the concentration and nature of both the

protein and the drug, pH, and temperature.

The extent of binding of antibiotics has been measured most

frequently either by dialysis, as for example by Kunin (46-50), Tompsett

et al. (90), and Smith et al. (86), or by ultrafiltration as performed

by Sirota and Slatzman (82), Verwey and Williams (96), Keen (36) and

Rolinson and Sutherland (74). It may be well to note that both of

these physical principles operate in vivo (73).

At the levels generally encountered in the body the extent of

binding of antibiotics is almost independent of concentration. Binding,

however, decreases with increased concentration in excess of $10^{-4}$M at

which point the majority of the protein-binding sites are occupied, as

stated by Rolinson and Sutherland (74).

Rolinson (73) calls attention to the fact that it is the proportion

of free drug which is important therapeutically, a point of view also

expressed by Goldstein (25) in his statement that "Microbiological

techniques permit direct proof that the potency of an antibacterial

drug in the presence of protein is exacly that of its unbound fraction".

Because of the dynamic nature of protein-antibiotic interaction,

movement of free antibiotic in the absence of any unusual barrier takes

place throughout the extracellular fluid volume of the body wherever a

concentration gradient exists.  The outcome of such a distribution is a

tendency to equilibrium, and although the levels of total antibiotic may

be different in the various tissues, the level of free, unbound anti-

biotic will be the same.  This latter concept is enunciated by Scholtan

and Schmid (78) in the statement:

> ....the various organs may show different
> drug content because of different binding
> ability of the protein in the organ; on
> the other hand, independently of this,
> after achievement of equilibrium the con-
> centration of free drug in the tissues and
> fluids of all compartments in the body must
> be equal and identical with the free con-
> centration in the plasma fluids ...

Some drugs are, however, unable to get into every fluid compartment of

the body; for example, as stated by Goodman and Gilman (26), penicillin

does not pass the blood-brain barrier.  Other antibiotics such as

chloramphenicol, as shown by Glazko et al. (24) are rapidly inactivated

and excreted.

Diagrammatic representation of the distribution of drugs

according to the principles defined by Rolinson (72) are presented in

Fig. I.

Most of the studies on antibiotic binding have used human or

horse sera.  It is important therefore that attention be drawn to the
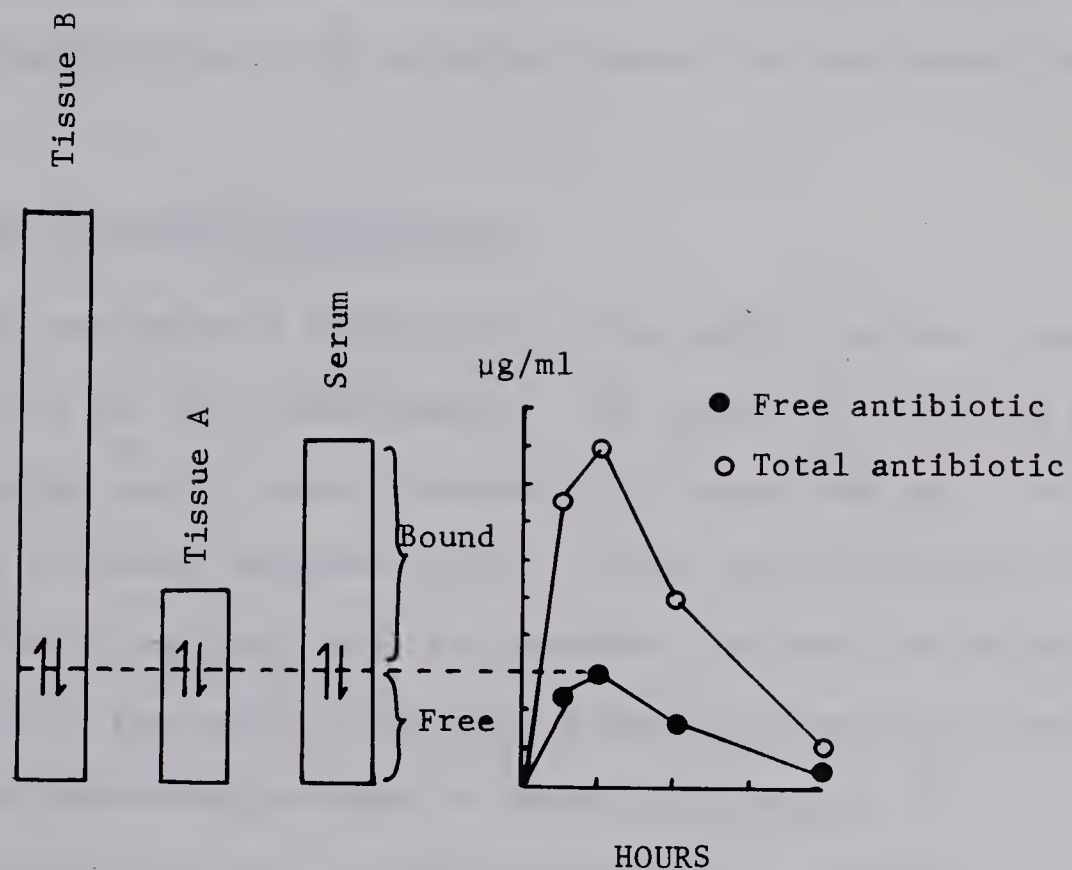
FIGURE 1. Diagrammatic representation of the influence of protein binding on serum and tissue levels of antibiotic.

(Rolinson, 1964. Postgrad. med. J. Suppl. 40:20-22.)

fact that there is great variation in the extent of binding of any given

antibiotic in the sera of different animal species (73). In addition,

as pointed out by Verwey and Williams (96) the relative binding of

different antibiotics in the serum may change from one animal species to

another.

Mechanisms of action of antibiotics.

The mechanism of antimicrobial drug action has been a subject of

consideration for many investigators. The subject is certainly pertinent

to the present study, however because of its magnitude only a brief

summary of currently accepted points of view, with particular focus on

the antibiotics employed, will be presented. An excellent source of

information is the recently concluded Sixteenth Symposium of the Society

for General Microbiology edited by Newton and Reynolds (63).

As indicated in a review by Newton (62), antibiotics can be

placed in one or more broad categories according to their mode of action,

viz.:

1.      Antibiotics affecting cell-wall synthesis.

2.      Antibiotcs affecting cell-membrane synthesis.

3.      Antibiotics affecting cell-membrane permeability.

4.      Uncoupling agents and inhibitors of electron transport.

5.      Inhibitors of nucleotide or of DNA synthesis.

6.      Antibiotics affecting the transcription of genetic information.

7.      Inhibitors of protein synthesis.

Of the antibiotics used in this study, penicillin, bacitracin,

D-cycloserine, oxacillin and vancomycin belong to the category of those mainly affecting cell-wall synthesis, whereas chloramphenicol, as shown by Gale and Paine (23) and Gale and Folkes (22), inhibits protein synthesis.

The current concept of the reactions involved in the formation of murein, the backbone structure of all bacterial cell walls, is presented in Fig. 2 and Fig. 3, from Park (66). D-cycloserine is a competitive inhibitor of D-ala-D-ala ligase and prevents the incorporation of the dipeptide in the pathway leading to the formation of UDP-NAc-muramyl-pentapeptide as demonstrated by Strominger, Ito, and Threnn (89). The work of Anderson et al. (3) demonstrated that bacitracin and vancomycin interfere with the transfer of disaccharide-pentapeptide from phospholipid acceptor to form oligomuropeptide. The report of Wise and Park (97) would seem to indicate the penicillin inhibits the cross-linking reactions involved in the synthesis of murein.

Pyrimidine nucleotide precursors

UTP

ADP
ATP

UDP

UDP - NAc + glucosamine

phosphoenolpyruvate

UDP - NAc - muramic acid

L-Alanine, ATP, $Mn^{2+}$

UDP - NAc - muramyl —— L-ala

D-Glutamic acid, ATP, $Mn^{2+}$

UDP - NAc - muramyl —— L-ala —— D-glu

L-Lysine, ATP $Mn^{2+}$

UDP - NAc - muramyl —— L-ala —— D-glu —— L-lys.

ATP
$Mn^{2+}$

D-ala $\frac{ATP}{Mn^{2+}}$ → D-ala —— D-ala

UDP - NAc-muramyl —— L-ala —— D-glu —— ($\gamma$) —— L-lys —— D-ala —— D-ala
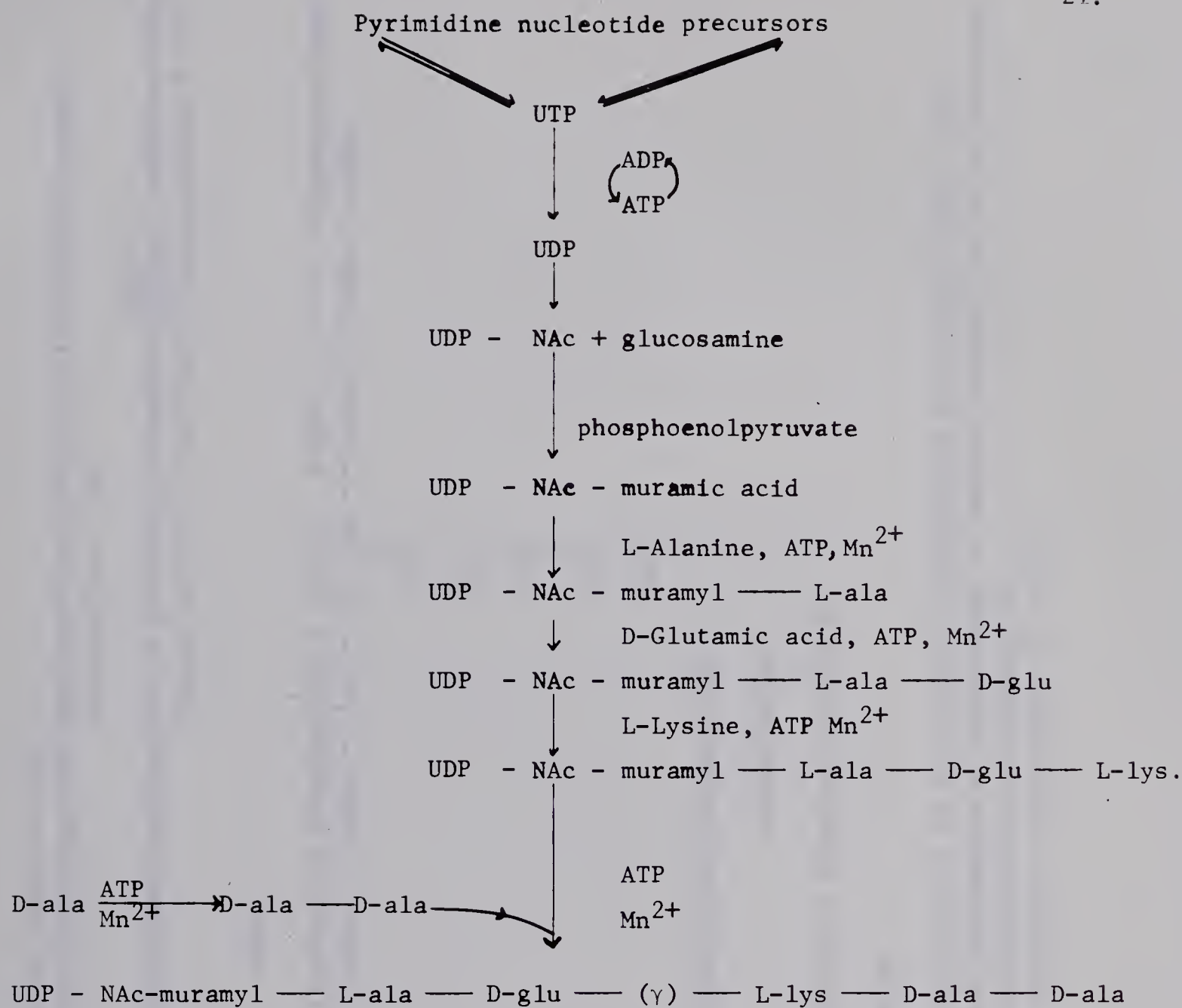
FIGURE 2.  Reactions leading to the synthesis of UDP-NAc-muramyl
pentapeptide.

(Park, 1966.  Soc. for Gen. Microbiol. 16th Symp. 70-81.)

(1)  UDP-NAc-muramyl-pentapeptide $\xrightarrow{\text{membrane particles Mg}^2}$ membrane-bound phospholipid*-P-P-NAc-muramyl-
     pentapeptide + UMP

(2)  Membrane-bound phospholipid-P-P-NAc-muramyl-pentapeptide + UDP-NAc-glucosamine $\xrightarrow{\text{Mg}^{2+}}$ membrane-bound
     phospholipid-P-P-disaccharide-pentapeptide + UDP

(3)  Glycine + s-RNA $\xrightarrow{\text{ATP Mg}^{2+}}$ glycyl   s-RNA

(4)  Membrane-bound phospholipid-P-P-disaccharide pentapeptide + glycyl   s-RNA $\longrightarrow$ membrane-bound
     phospholipid-P-P-disaccharide —— L-ala —— D-glu —— (γ) —— L-lys —— D-ala ——D-ala + s-RNA
                                                                          |ε
                                                                          gly
                                                                          |
                                                                          gly
                                                                          |
                                                                          gly
                                                                          |
                                                                          gly
                                                                          |
                                                                          gly

(5)  Membrane-bound phospholipid-P-P-disaccharide-pentapeptide
                                                  |ε
                                                  pentaglycine

     $\xrightarrow{\text{Mg}^{2+}}$ (disaccharide-pentapeptide)η + phospholipid-P + $P_i$
                                    |ε
                                    pentaglycine

FIGURE 3.   Reactions leading to the incorporation of NAc-muramyl-pentapeptide into murein which lacks
            cross-links. *Phospholipid-P- represents a phospholipid of unknown composition in the membrane
            whose phosphate accepts phospho-NAc-muramyl-pentapeptide.
            (Park, 1966.  Soc. for Gen. Microbiol., 16th Symp.:70 – 81.)

II

THE GROWTH OF <u>STAPHYLOCOCCUS</u> <u>AUREUS</u> PS7

AND STAPHYLOPHAGE 7, AND THE INFLUENCE

OF ANTIBIOTICS ON THE LYSIS OF <u>S</u>. <u>AUREUS</u>

PS7 BY Ø7; <u>IN</u> <u>VITRO</u>.

II

## MATERIALS AND GENERAL METHODOLOGY.

A brief account of the source of the materials used in this study is presented below. Also included are those aspects of general methodology common to many of the experiments performed; modifications and other special techniques are discussed wherever warranted.

Media.

The nutrient medium employed was 2.5% nutrient broth (Oxoid #2) (= NB). The broth was sterilized at $121^{\circ}$ for 15 minutes and the pH on cooling was ca. 7.2. When nutrient medium supplemented with $Ca^{++}$ was needed, sterile stock $CaCl_2$ solution was added to sterile broth to give the final concentration required, usually 100 µg/ml $CaCl_2$ (= $NBCa^{++}$).

Nutrient agar for general use was prepared by adding 1.5% agar (Bacto Difco) to nutrient broth before sterilization. For use in the assay of phage by the agar overlay technique of Gratia (28), 0.5% agar was added to NB.

Bacterial strain.

Staphylococcus aureus PS7 in the lyophilized state, originating from the Department of National Health and Welfare, was obtained through the courtesy of the phage-typing section of the Provincial Laboratory of Public Health, University of Alberta, and transfers were made at ca. 2-month intervals.

The phage type of S. aureus PS7 was 6/7/47/53/54/75/77 and it remained unchanged throughout this investigation.

## Preparation of bacterial suspensions

When log-phase suspensions of S. aureus PS7 were required,
growth from a stock NA slope was spread on NA plates and incubated
overnight at 37$^\circ$C. Growth from each of one or more plates was harvested
using ca. 2 - 5 ml NB (or NBCa$^{++}$) and adjusted to an absorbance of 1.0.
This latter suspension was then used to inoculate NB (or NB Ca$^{++}$),
(1 ml suspension:100 ml nutrient broth), and the organisms were then
grown to the turbidity required for use in a water-bath shaker at 37$^\circ$C.

## Bacteriophage

Staphylococcus bacteriophage 7 was obtained in the lyophilized
state from the same source as S. aureus PS7. After reconstitution it
was propagated on S. aureus PS7 in NB (later NB Ca$^{++}$ was used) by the
method described by Blair and Williams (10). Lysates of Ø7 after
propagation and filtration through millipore 0.45μ HA membrane filters
contained ca. $10^9 - 10^{10}$ pfu/ml. For the preparation of high titer
phage, filtered lysates were centrifugalized at 18,000 rpm for 6 - 8
hours in a Serval RC-2 centrifuge. Upon removal of most of the super-
natant, pooled concentrated suspensions gave titers of ca. 1 - 1.5 x
$10^{11}$ pfu/ml.

## Viable counts

For the determination of viable counts, suspensions of bacteria
were diluted in 0.07 M phosphate buffer at pH 7.0, prepared as described
by Varley (94), and 0.1 ml volumes of appropriate dilutions were then

spread on the dried surfaces of NA plates.  After 24 hours incubation at 37°C colonies were counted with the aid of a New Brunswick Bactronic Scientific counter.  Counts were recorded from plates showing 30 - 300 colonies.

## Measurement of absorbance

Absorbance of bacterial suspensions was determined with the aid of a Bausch & Lomb Spectronic 20 spectrometer using a wave-length of 600 mμ .  Suspensions were contained in side-arm flasks and were tipped into the side-arms for reading.

## Antibiotic sensitivity of S. aureus PS7.

Antibiotic sensitivity tests by the disc technique showed that S. aureus PS7 is susceptible to vancomycin, novobiocin, chloramphenicol, bacitracin, streptomycin, penicillin and oxacillin.  Sensitivity to D-cycloserine was demonstrated using a tube technique.

## Antibiotics

The antibiotics used in this investigation and their origin are listed in Appendix I.  Stock antibiotic solutions were made by weighing appropriate amounts of each antibiotic and dissolving them in water. The concentrations reported are those of the solid material supplied by the manufacturers.

GROWTH OF STAPHYLOCOCCUS AUREUS PS7

AND OF Ø7.

Standard growth of S. aureus PS7 in nutrient broth at 37°C.

A standard growth curve for Staphylococcus aureus PS7 was

determined by using a suspension of S. aureus PS7 of absorbance 1.0,

prepared from overnight growth on an NA plate. A 1% inoculum in nutrient

broth was made in a final volume of 50 ml contained in a 250 ml side-arm

flask. The flask (with its contents) was incubated at 37°C in a water-

bath shaker. The absorbance of the suspension was determined at

intervals and at the same time 0.5 ml samples were removed and assayed

for viable organisms. Counts from three plates were recorded at each

time interval.

The results are presented in Table I, and are represented

graphically in Fig. 4.

The computed growth rate of S. aureus PS7 in the log phase under

these conditions is ca. 2 generations per hour.

The influence of antibiotics on absorbance of suspensions of

S. aureus PS7 at 37°C.

Log phase suspensions of S. aureus PS7 were subjected to the

influence of various concentrations of each of the antibiotics, penicillin,

D-cycloserine, bacitracin, vancomycin, chloramphenicol, and oxacillin.

The staphylococci and antibiotic were contained in a final volume of

50 ml in 250 ml side-arm flasks. The incubation temperature was 37°C,

TABLE I

## GROWTH OF STAPHYLOCOCCUS AUREUS PS7

### IN NUTRIENT BROTH AT 37°C.

| At Hrs. | Absorbance | 0.1 ml of dilution plated | Counts/plate | Standard error | cfu/ml |
|---|---|---|---|---|---|
| 0 | .002 | $10^3$ | 50,48,60 | 3.71 | $5.27 \times 10^5$ |
| ½ | .019 | $10^3$ | 57,66,50 | 4.63 | $5.77 \times 10^5$ |
| 1 | .023 | $10^3$ | 86,75,70 | 4.73 | $7.70 \times 10^5$ |
| 2 | .028 | $10^3$ | 146,150,160 | 5.77 | $1.50 \times 10^6$ |
| 3 | .035 | $10^4$ | 69,65,62 | 2.28 | $6.53 \times 10^6$ |
| 4 | .096 | $10^5$ | 37,30,37 | 5.32 | $3.40 \times 10^7$ |
| 5 | .390 | $10^5$ | 188,196,200 | 3.53 | $1.94 \times 10^8$ |
| 6 | .76 | $10^6$ | 34,32,30 | 1.15 | $3.20 \times 10^8$ |
| 7 | 1.00 | $10^6$ | 77,68,71 | 2.65 | $7.20 \times 10^8$ |
| 8 | 1.20 | $10^6$ | 77,79,83 | 1.76 | $7.97 \times 10^8$ |
| 9 | 1.35 | $10^6$ | 78,82,74 | 2.31 | $7.80 \times 10^8$ |
| 10 | 1.40 | $10^6$ | 72,75,86 | 4.26 | $7.70 \times 10^8$ |

S. aureus PS7 suspension of absorbance 1.0 was used to inoculate NB (1 in 100), and incubated at 37°C. Absorbance and viable counts were determined at intervals.
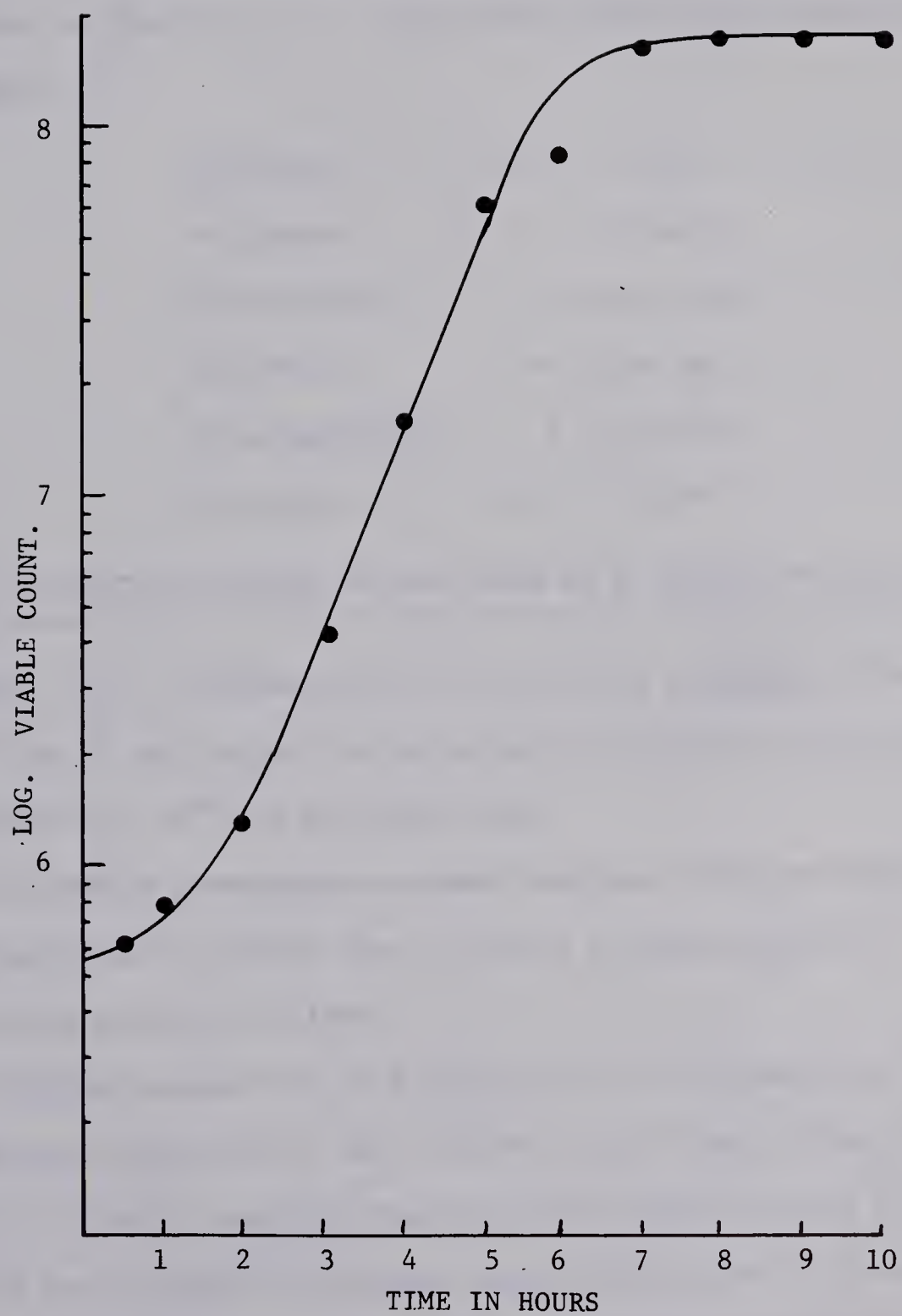
FIGURE 4. Growth of <u>S</u>. <u>aureus</u> PS7 in nutrient broth at 37°C.

and the turbidities were read at half-hour intervals.

Results typical of the antibiotics used against S. aureus PS7 are presented in Fig. 5 (a - f). The minimal inhibitory concentrations observed were:

| | |
|---|---|
| Penicillin | 0.1 - 1 µg/ml |
| Vancomycin | 1 - 10 µg/ml |
| D-cycloserine | 10 - 100 µg/ml |
| Bacitracin | 10 - 100 µg/ml |
| Chloramphenicol | 1 - 10 µg/ml |
| Oxacillin | 0.2 - 2 µg/ml |

## Influence of $CaCl_2$ (120 µg/ml) on the lysis of S. aureus PS7 by Ø7

After much unrewarded effort using Ø7 and S. aureus PS7 in nutrient broth it was decided to investigate the extent to which this system depended on $Ca^{++}$ for efficient lysis.

Preliminary investigation showed that when $CaCl_2$ was added to NB to a concentration greater than 350 µg/ml a precipitation of insoluble calcium salts resulted.

Log-phase suspensions of S. aureus PS7 of absorbance ca. 0.4 with and without added $CaCl_2$, were infected with Ø7 and allowed to incubate at 37°C with shaking. The phage:cell ratios studied were ca. 15:1, 15:10 and 15:100. The mixtures were contained in 40 ml volumes in 250 ml side-arm flasks. The absorbances of the suspensions were determined at intervals.
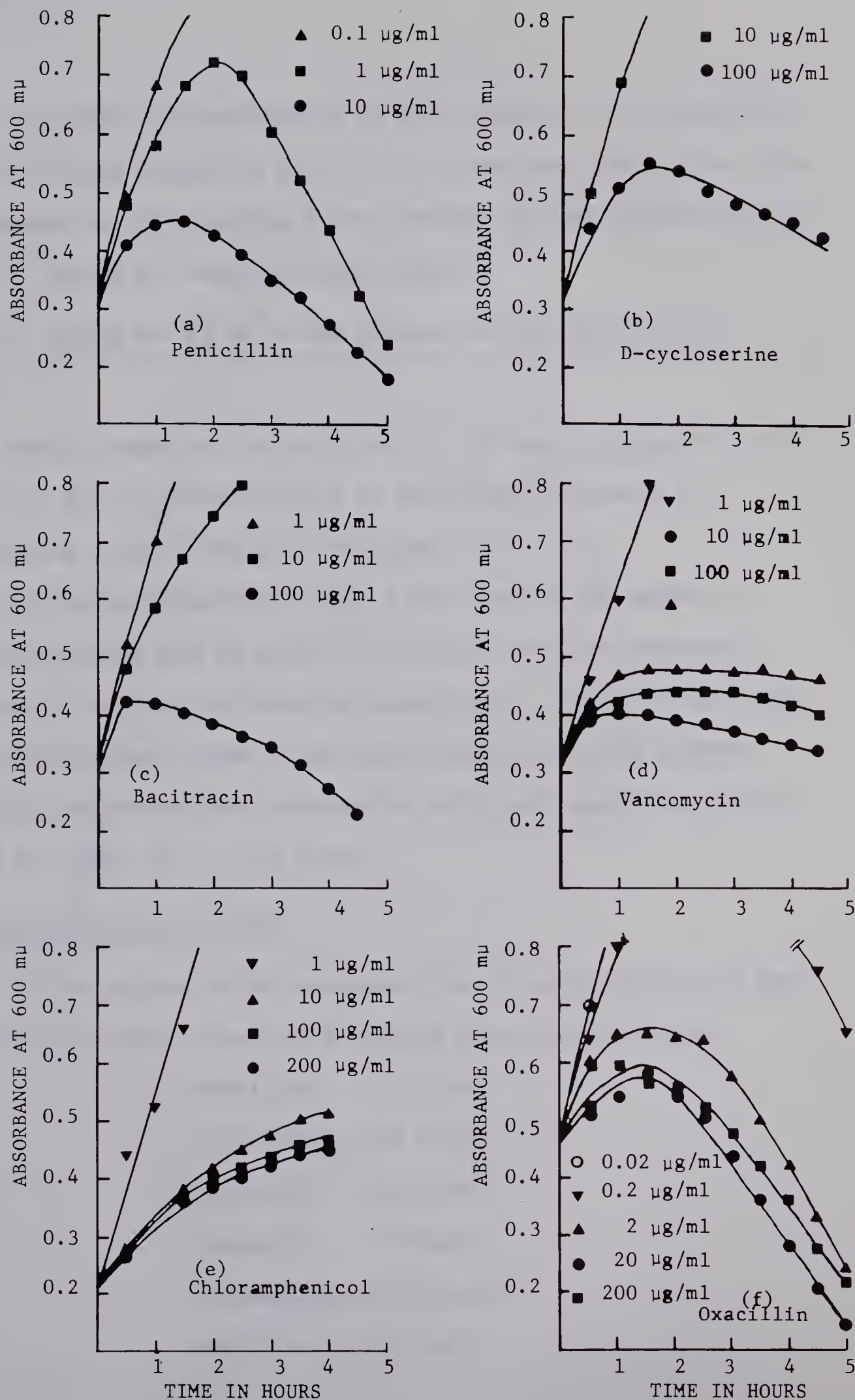
FIGURE 5. The influence of antibiotics on absorbance of suspensions of
S. aureus PS7 at 37°C.

The results are represented in Fig. 6 where it can be seen that even with the high phage:cell ratio of 15:1 there was little or no lysis in the absence of $Ca^{++}$, whereas in the presence of this cation efficient lysis of S. aureus PS7 suspension was evident.

## Lysis of S. aureus PS7 by Ø7 in the presence of different amounts of $CaCl_2$

Using a phage:cell ratio of ca. 6:1, Ø7 and S. aureus PS7 were incubated at $37^{o}C$ in nutrient broth to which $CaCl_2$ was added in concentrations ranging from 20 - 120 μg/ml.

The results presented in Fig. 7 show that as the amount of $CaCl_2$ was increased from 20 μg/ml to 75 μg/ml, there was increased efficiency in the lysis of bacterial suspensions. Beyond 75 μg/ml the rate of lysis did not appear to be significantly augmented further. This latter observation was confirmed by additional experiments using $CaCl_2$ in the range of 50 - 120 μg/ml.

## Influence of antibiotics on Ø7

To 9 ml volumes of Ø7 suspension 1 ml of stock solution of each antibiotic was added to give the following final concentrations:

Penicillin,      10 μg/ml

D-cycloserine,100 μg/ml

Bacitracin,     100 μg/ml

Vancomycin      10 μg/ml

Chloramphenicol, 20 μg/ml

Oxacillin,       20 μg/ml
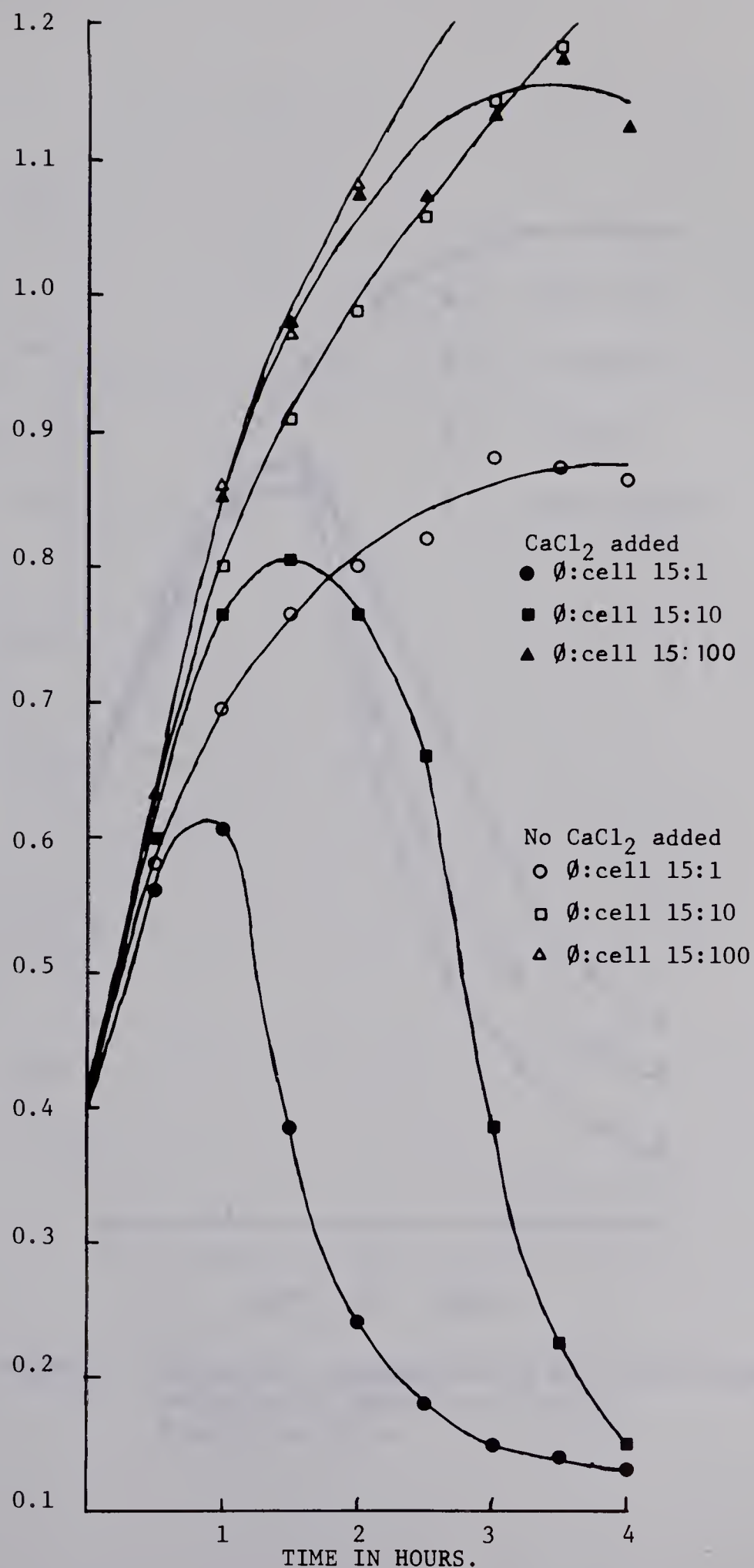
FIGURE 6. Influence of $CaCl_2$ (120 μg/ml) on the lysis of S. aureus PS7 by ∅7.
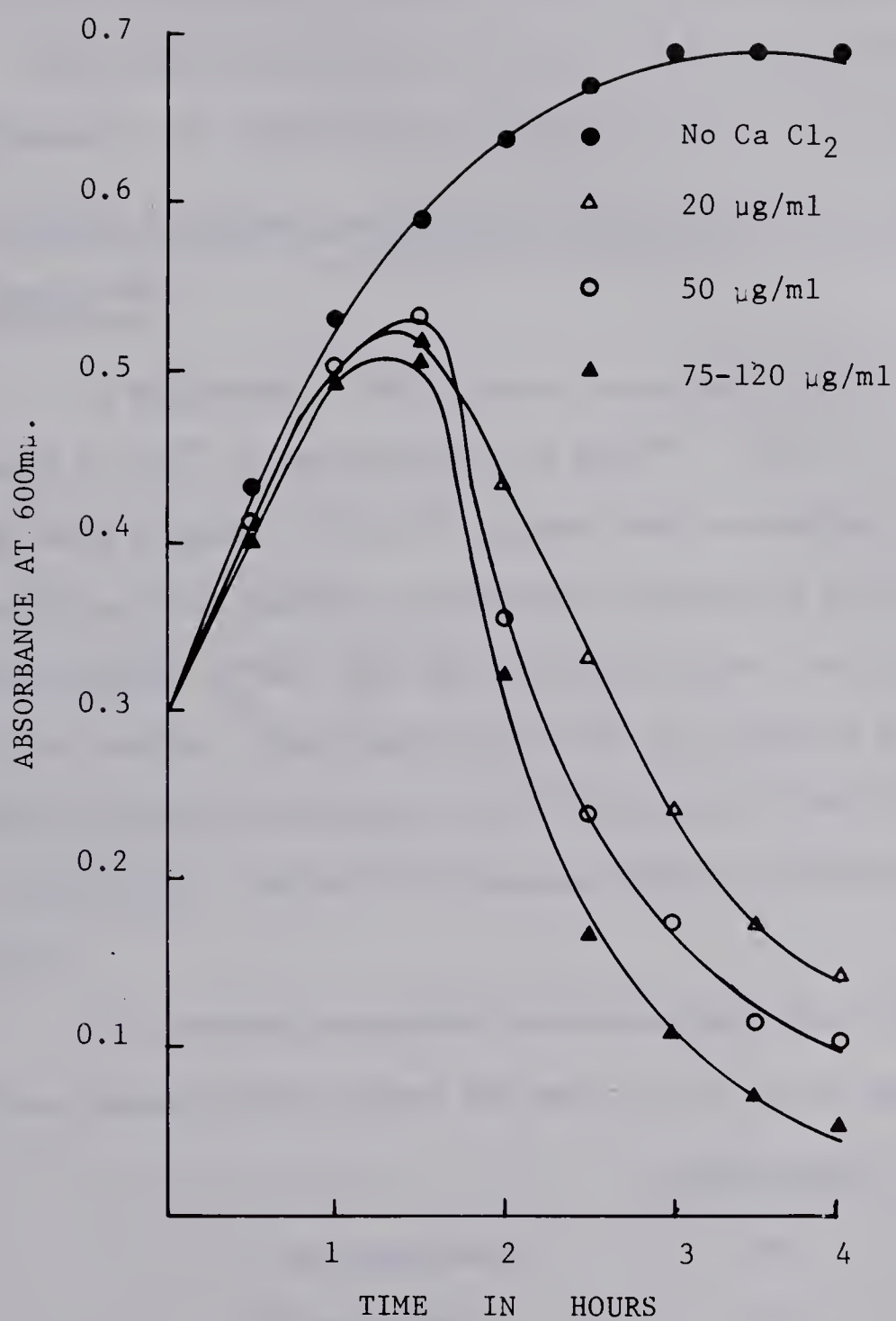
FIGURE 7.  Lysis of S. aureus PS7 by Ø7 in the presence
of different amounts of Ca Cl$_2$.
Ø:cell, ca. 6:1.

The number of plaque-forming units was determined immediately after addition of antibiotic and again after 48 hours storage in a cold room at $4^{o}$C. The results presented in Table II show that there was no significant influence of the antibiotics on the assay value of Ø7 suspension.

The effect of residual antibiotic on the assay of Ø7 against

S. aureus PS7

A suspension of Ø7 of assay value ca. 2.5 x $10^9$ pfu/ml was diluted to $10^{-6}$ in tenfold steps in NBCa$^{++}$. Five other sets of dilutions of Ø7 were prepared, the $10^{-3}$ diluent tube containing the antibiotics penicillin, D-cycloserine, bacitracin, vancomycin and chloramphenicol in concentrations of 10, 100, 100, 10 and 20 µg/ml, one antibiotic to each dilution series. The final tube ($10^{-6}$) was used for preparing the agar overlay plates and contained a $10^{-3}$ dilution of the initial concentration of antibiotics. The set of dilutions without antibiotic served as a control.

The results presented below show that the residual antibiotic did not significantly affect the assay of Ø7 vs. S. aureus PS7.

|  | x $10^9$ pfu/ml |
| --- | --- |
| No antibiotic | 1.26 |
| Penicillin | 1.32 |
| D-cycloserine | 1.27 |
| Bacitracin | 1.24 |
| Vancomycin | 1.27 |
| Chloramphenicol | 1.30 |

## TABLE II

### INFLUENCE OF ANTIBIOTICS ON ∅7

|  | Zero time (x $10^9$ pfu/ml) | 48 hrs. at $4^{o}C$ (x $10^9$ pfu/ml) |
|---|---|---|
| Control (no antibiotic) | 7.95 | 8.17 |
| Penicillin, 10 µg/ml | 8.15 | 8.07 |
| D-cycloserine, 100 µg/ml | 8.10 | 8.02 |
| Bacitracin, 100 µg/ml | 8.13 | 8.18 |
| Vancomycin, 10 µg/ml | 8.12 | 8.03 |
| Chloramphenicol, 20 µg/ml | 7.94 | 8.11 |
| Oxacillin, 20 µg/ml | 8.20 | 8.23 |

To 9 ml of ∅7 suspension 1 ml of antibiotic solution was added to give the antibiotic concentrations indicated.  The suspensions were assayed for plaque forming units immediately after addition of antibiotic and again 48 hours after storage at $4^{o}C$.

Adsorption of Ø7 to <u>S. aureus</u> PS7 at 37°C and the effect of antibiotics

on the adsorption process

    The experiment reported below served to determine the adsorption

characteristics of Ø7 to <u>S. aureus</u> PS7, as well as to reveal the extent

to which antibiotics influenced such adsorption.

    Phage 7 was added to log-phase <u>S. aureus</u> PS7 suspensions at

37°C to give a final concentration of $6.80 \times 10^6$ pfu/ml.  The viable

count on the staphylococcal suspension was $2.70 \times 10^8$ cfu/ml giving a

Ø:cell ratio of 1:40.  Where investigated, the appropriate antibiotic

was added 1 minute before the bacteriophage to give final concentrations

as follows:

|  |  |
|---|---|
| Penicillin | 10 µg/ml |
| D-cycloserine | 100 µg/ml |
| Bacitracin | 100 µg/ml |
| Vancomycin | 10 µg/ml |
| Chloramphenicol | 20 µg/ml |

At 2½. 5, and 10 minute intervals after addition of Ø7, 0.5 ml of each

adsorption mixture was removed and diluted $10^{-2}$ in cold (ice water bath)

NBCa$^{++}$.  After further 10-fold dilution, 5 ml portions were removed and

centrifugalized at 3000 rpm for 15 minutes.  The supernatant from each

tube was carefully removed and assayed for residual Ø7.

    Results are presented in Table III and the adsorption curve for

Ø7 vs. <u>S. aureus</u> PS7 in the absence of antibiotic is represented in

Fig. 8.  It can be seen that 85% of Ø7 adsorbs in 2½ minutes, ca. 95%

TABLE III

ADSORPTION OF Ø7 TO S. AUREUS PS7 AT 37°C AND THE

EFFECT OF ANTIBIOTICS ON THE ADSORPTION PROCESS

| | Time min. | P | % residual Ø7 | % adsorbed Ø7 | K, (x $10^{-9}$ ml/min) |
|---|---|---|---|---|---|
| No antibiotic | 2.5 | $1.02 \times 10^6$ | 14.9 | 85.1 | 2.819 |
| | 5 | $3.63 \times 10^5$ | 5.3 | 94.7 | 2.171 |
| | 10 | $1.08 \times 10^5$ | 1.6 | 98.4 | 1.533 |
| Penicillin 10 µg/ml | 2.5 | $1.02 \times 10^6$ | 14.9 | 85.1 | 2.819 |
| | 5 | $4.03 \times 10^5$ | 5.9 | 94.1 | 2.094 |
| | 10 | $1.02 \times 10^5$ | 1.5 | 98.5 | 1.554 |
| D-cycloserine 100 µg/ml | 2.5 | $9.40 \times 10^5$ | 13.7 | 86.3 | 2.936 |
| | 5 | $4.10 \times 10^5$ | 6.0 | 92.0 | 2.081 |
| | 10 | $1.03 \times 10^5$ | 1.5 | 98.5 | 1.542 |
| Bacitracin 100 µg/ml | 2.5 | $1.06 \times 10^6$ | 15.5 | 84.5 | 2.758 |
| | 5 | $4.10 \times 10^5$ | 6.0 | 92.0 | 2.081 |
| | 10 | $9.90 \times 10^4$ | 1.4 | 98.6 | 1.565 |
| Vancomycin 10 µg/ml | 2.5 | $6.91 \times 10^5$ | 10.1 | 89.9 | 3.019 |
| | 5 | $3.23 \times 10^5$ | 4.7 | 94.3 | 2.264 |
| | 10 | $7.22 \times 10^4$ | 1.1 | 98.9 | 1.700 |
| Chloram- penicol 20 µg/ml | 2.5 | $6.71 \times 10^5$ | 9.8 | 90.2 | 3.437 |
| | 5 | $3.10 \times 10^5$ | 4.7 | 95.3 | 2.264 |
| | 10 | $6.09 \times 10^4$ | 0.9 | 99.1 | 1.744 |

$$K = \frac{2.3}{B} \times \frac{1}{t} \times \log \frac{Po}{p}$$

Po = initial concentration of Ø7
P  = concentration of Ø7 at time t
t  = time in minutes
B  = concentration of bacterial cells (cfu/ml)

in 5 minutes and close to 100% in 10 minutes. The presence of any of the tested antibiotic 1 minute before $\emptyset$7 and during the adsorption period did not have any noticeable effect on the rate or extent of adsorption to S. aureus PS7. It is also evident from the slope of the curve in Fig. 8 and from adsorption constants (K) computed on the basis of residual $\emptyset$7 at 2½. 5. and 10 minutes, that the final fractions of $\emptyset$7, ca. 15%, adsorb more slowly than the first 85% representing the bulk of the added $\emptyset$7.

## One-step growth of $\emptyset$7 using S. aureus PS7 as host

The method used for investigating one-step growth was that of Ellis and Delbruck (19). From an adsorption mixture of $\emptyset$7 and log phase S. aureus PS7 at 37°C 0.5 ml samples were removed after 5 minutes and diluted $10^{-2}$ in NBCa$^{++}$ at 37°C. Incubation at 37°C was continued in the water-bath shaker. At intervals 0.5 ml of the dilute incubating mixture was removed and further diluted for assay of total infectious centers. For these latter dilutions NBCa$^{++}$ at the temperature of an ice water bath was used.

The results of a sample experiment are presented in Fig. 9. The S. aureus PS7 suspension contained 2.70 x $10^8$ cfu/ml and the $\emptyset$:cell ratio was 1:40. Under these conditions a latent period of ca. 30 minutes was obtained. The rise period was ca. 22 minutes and the burst size ca. 95.

## The effect of antibiotics on the yield of $\emptyset$7 using S. aureus PS7 as host at 37°C.
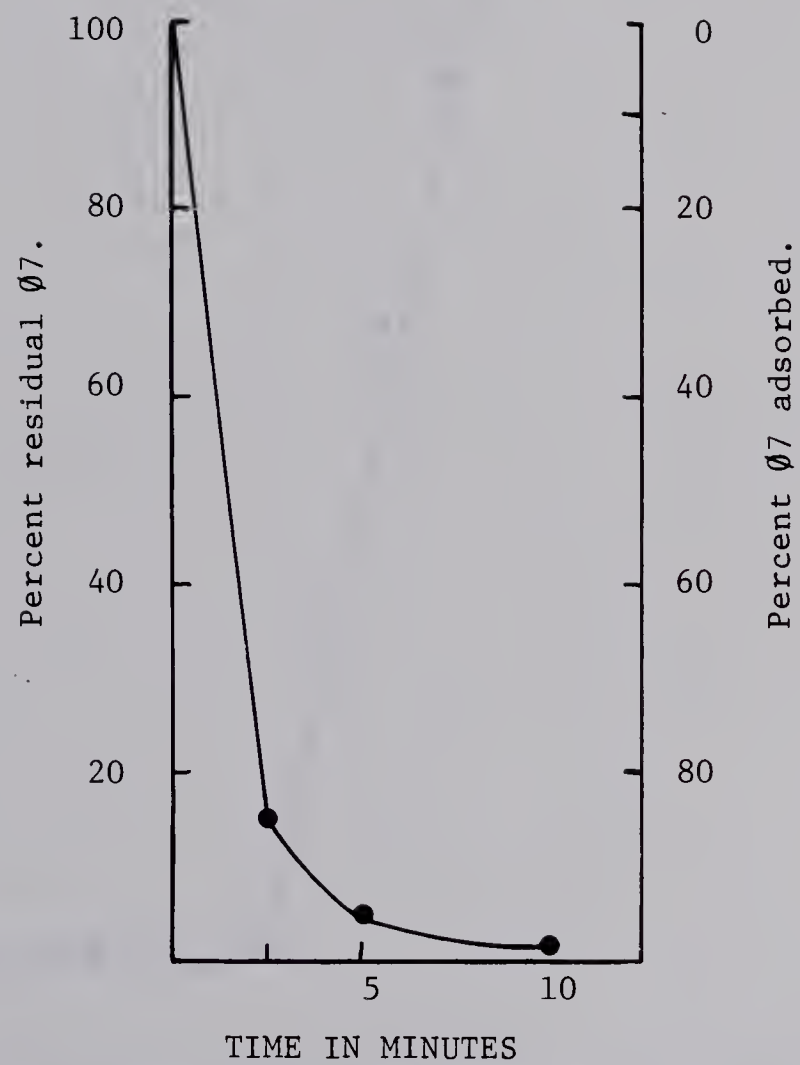
FIGURE 8. Adsorption of Ø7 to S. aureus PS7 at 37°C.
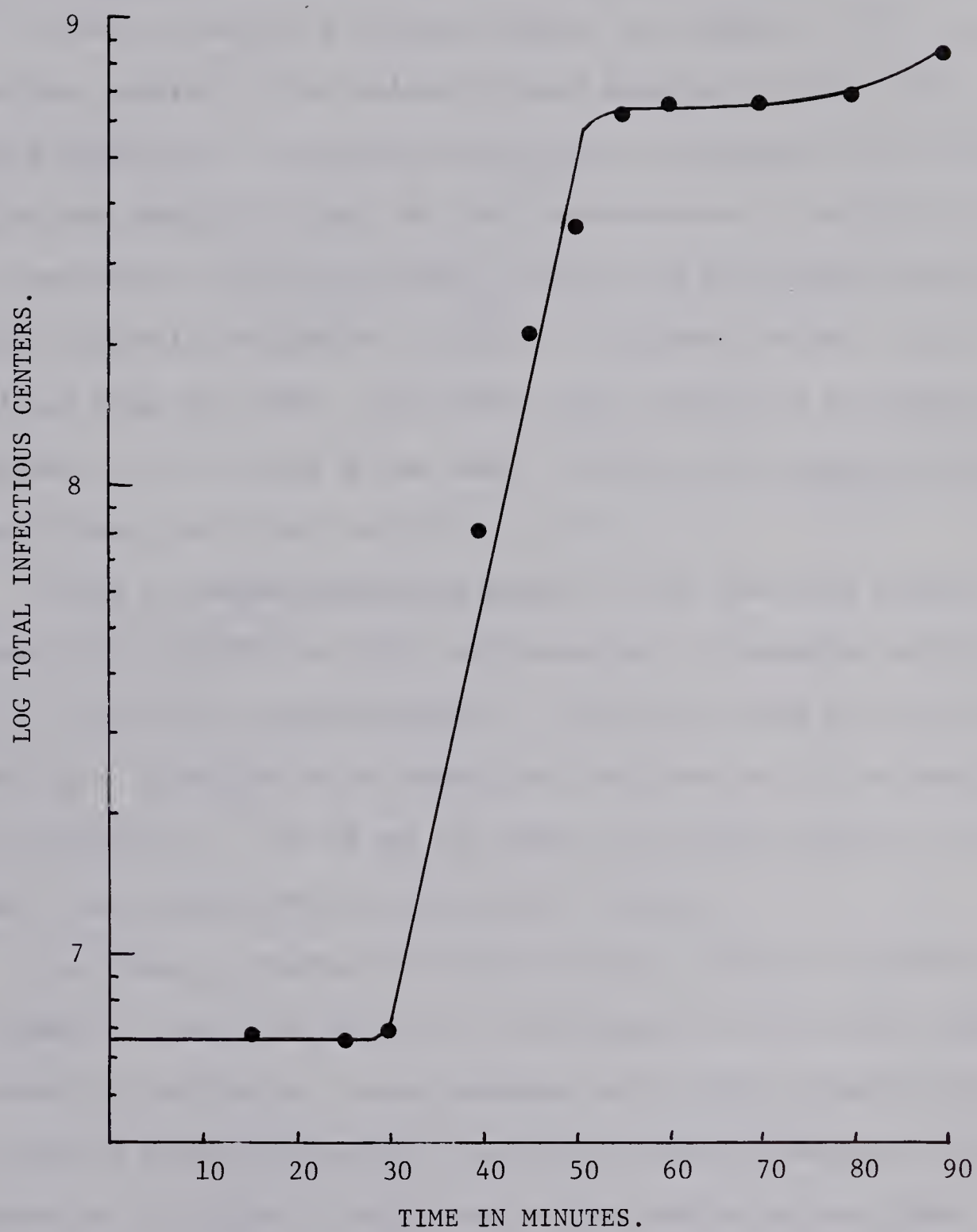
FIGURE 9.   One-step growth of Ø7 using <u>S</u>. <u>aureus</u> PS7 as host.

S. aureus PS7 suspension in the log phase was distributed in
48 ml volumes to each of 6 side-arm flasks, and warmed to 37°C  in a
water-bath shaker.  A 1 ml volume of stock solution of each of the anti-
biotics penicillin, D-cycloserine, bacitracin, vancomycin, and chloram-
penicol was added, such that the final concentration of antibiotic in
50 ml would be 10 µg, 100 µg, 100 µg, 10 µg, and 20 µg per ml respectively.
To one flask which served as a control, a comparable volume, 1 ml of
distilled water was added.  One minute after addition of the antibiotic
(or water), 1 ml of stock Ø7 was used to infect the S. aureus suspension
in each flask; the Ø:cell ratio was ca. 1:12.

After a 5-minute adsorption period, 0.5 ml from each flask was
diluted $10^{-2}$ in NB $Ca^{++}$ at 37°C containing the corresponding antibiotic
at the concentration indicated above.  The control flask did not contain
antibiotic.  Incubation with shaking was continued at 37°C and samples
were withdrawn at 7, 20, 60 and 120 minute intervals, diluted in cold
NB $Ca^{++}$, and assayed for total infectious centers.

The results presented in Table IV show a 140-fold increase in
the number of infectious centers at 120 minutes for the control which
contained no antibiotic.  In the presence of 10 µg/ml of penicillin,
100 µg/ml of D-cycloserine and 10 µg/ml of vancomycin there was also an
increase in the number of infectious centers indicating that these
antibiotics had allowed both Ø7 replication and release of active Ø7.
The increased titre in these three systems was not, however, as high as
in the absence of antibiotic.  In the presence of 10 µg/ml of

TABLE IV

THE EFFECT OF ANTIBIOTICS ON THE YIELD OF

Ø7 USING S. AUREUS PS7 AS HOST AT 37°C

Minutes after Ø7 addition

| | 7 pfu/ml | 30 pfu/ml | 60 pfu/ml | 120 pfu/ml |
|---|---|---|---|---|
| Control (no antibiotic) | $2.54 \times 10^7$ | $3.20 \times 10^7$ | $2.28 \times 10^8$ | $3.55 \times 10^9$ |
| Penicillin, 10 µg/ml | $2.54 \times 10^7$ | $2.06 \times 10^7$ | $4.46 \times 10^7$ | $4.68 \times 10^7$ |
| D-cycloserine, 100 µg/ml | $2.13 \times 10^7$ | $2.27 \times 10^7$ | $7.06 \times 10^7$ | $7.5 \times 10^7$ |
| Bacitracin, 100 µg/ml | $1.22 \times 10^7$ | $3.50 \times 10^6$ | $1.22 \times 10^7$ | $1.23 \times 10^7$ |
| Vancomycin, 10 µg/ml | $1.86 \times 10^7$ | $1.30 \times 10^7$ | $8.83 \times 10^7$ | $1.16 \times 10^8$ |
| Chloramphenicol, 20 µg/ml | $2.83 \times 10^7$ | $2.42 \times 10^7$ | $1.79 \times 10^7$ | $1.73 \times 10^7$ |

Ø7 added 1 minute after antibiotic in the
the concentrations indicated was used to
infect log-phase S. aureus PS7 suspensions,
at 37°C. in the ratio of 1:12. Input Ø7
was $2.52 \times 10^7$ pfu/ml. Total infectious
centers were determined at intervals following
Ø7 addition.

chloramphenicol and 100 µg/ml of bacitracin, the number of infectious

centers ultimately recovered was less than or equal to the input of

Ø7 respectively.

INTERACTION OF S. AUREUS PS7, Ø7 AND EACH OF THE
ANTIBIOTICS PENICILLIN, D-CYCLOSERINE,
BACITRACIN, VANCOMYCIN AND CHLORAMPHENICOL.

In these experiments S. aureus PS7 was grown at $37^O$C to the required absorbance of ca. 0.30 and was in the log phase. Unless otherwise stated, the nutrient medium was NBCa$^{++}$.

Prepared S. aureus PS7 suspensions were distributed in 48 ml volumes into 250 side-arm flasks. A 1 ml volume of Ø7 and 1 ml of antibiotic solution either alone or in combination were then added to each measured volume of cell suspension. When Ø7 or antibiotic were used alone, the volumes were made up to 50 ml with distilled water and nutrient broth respectively. The flasks were incubated at $37^O$C in a water bath shaker.

Absorbance values were recorded at intervals and are presented in Tables V - XVI. The maximum absorbance values (Amax), where appropriate, are recorded in separate columns. The time in hours required for suspensions to return to the absorbance values at the start of the experiments (ti) and the half-lysis time ($t\frac{1}{2}$) were obtained from graphs plotted on linear graph paper, and are also recorded in separate columns. The values in parentheses in the ti and $t\frac{1}{2}$ columns were estimated by extrapolation of the appropriate curves in the region of steepest negative slope. The Ø:cell ratios reported were based on assay of input Ø7 and the viable count of prepared cell suspensions at zero time.

The effect of different concentrations of penicillin.

S. aureus PS7 was subjected to the action of Ø7, and penicillin
at different concentrations; e.g. 10 µg/ml, 1 µg/ml, 0.1 µg/ml and
0.01 µg/ml; the Ø:cell ratio was ca. 1:1.

The results presented in Table V and Fig. 10 show that as the
concentration of antibiotic is decreased, the maximum turbidity obtained
by the suspensions before the onset of rapid lysis was increased. The
lytic patterns for S. aureus PS7 vs. Ø7 in the presence of 10 µg/ml,
1 µg/ml, 0.1 µg/ml and 0.01 µg/ml of penicillin were similar to that for
Ø7 alone. The respective half-lysis times, 1.9, 1.8, 1.7 and 2.5 hours
were less than that for Ø7 alone, viz., 2.9 hours. As the concentration
of penicillin was decreased, the curves intersected in the region of
rapid lysis in a manner indicating an increasing rate of rapid lysis as
the penicillin concentration was reduced from 10 µg/ml to 0.1 µg/ml.
This is also reflected in the decreasing half-lysis times, the values
becoming 1.9, 1.8 and 1.7 hours respectively.

Comparison of the effect of (a) the simultaneous addition of Ø7 and
penicillin, (b) Ø7 added ½ hour after penicillin and (c) penicillin
added ½ hour after Ø7.

In this experiment two concentrations of penicillin, 10 µg/ml
and 2 µg/ml, were investigated; the Ø:cell ratio was ca. 2:1.

Table VI and Fig. 11 show that when Ø7 was added at the same
time as penicillin or half an hour before penicillin, the curve for
lysis resembled that for Ø7 alone. Delay in the addition of penicillin

## TABLE V

### THE EFFECT OF DIFFERENT CONCENTRATIONS OF PENICILLIN

| S. aureus PS7 plus: | ABSORBANCE AT 600 mµ | | | | | | | | | | Amax | HOURS | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 hr. | ½ hr. | 1 hr. | 1½ hrs. | 2 hrs. | 2½ hrs. | 3 hrs. | 3½ hrs. | 4 hrs. | 4½ hrs. | | ti | t½ |
| Ø7 + pen.  10 µg/ml | 0.31 | 0.35 | 0.37 | 0.28 | 0.14 | 0.08 | 0.06 | 0.05 | 0.05 | 0.05 | 0.38 | 1.3 | 1.9 |
| Ø7 + pen.  1 µg/ml | 0.31 | 0.39 | 0.41 | 0.28 | 0.11 | 0.06 | 0.05 | 0.05 | 0.05 | 0.05 | 0.41 | 1.4 | 1.8 |
| Ø7 + pen.  0.1 µg/ml | 0.31 | 0.43 | 0.48 | 0.28 | 0.08 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.48 | 1.5 | 1.7 |
| Ø7 + pen.  0.01 µg/ml | 0.31 | 0.46 | 0.55 | 0.46 | 0.28 | 0.16 | 0.09 | 0.07 | 0.06 | 0.06 | 0.55 | 1.9 | 2.5 |
| Pen., 10 µg/ml | 0.31 | 0.36 | 0.39 | 0.39 | 0.37 | 0.33 | 0.28 | 0.24 | 0.19 | 0.15 | 0.39 | 2.8 | 4.3 |
| Pen.,  1 µg/ml | 0.31 | 0.41 | 0.43 | 0.44 | 0.42 | 0.38 | 0.33 | 0.28 | 0.22 | 0.17 | 0.44 | 3.1 | 4.6 |
| Pen.,  0.1 µg/ml | 0.31 | 0.44 | 0.55 | 0.64 | 0.66 | 0.62 | 0.52 | 0.43 | 0.32 | 0.22 | 0.66 | 4.0 | 4.8 |
| Pen.,  0.01 µg/ml | 0.31 | 0.48 | 0.64 | 0.77 | 0.82 | 0.87 | 0.87 | 0.87 | 0.81 | 0.85 | 0.87 | - | - |
| Ø7 only | 0.31 | 0.46 | 0.60 | 0.60 | 0.55 | 0.29 | 0.14 | 0.10 | 0.08 | 0.07 | 0.60 | 2.5 | 2.9 |
| S. aureus PS7 only | 0.31 | 0.49 | 0.69 | 0.85 | 1.00 | 1.13 | 1.30 | 1.35 | - | - | - | - | - |

Ø:cell ca. 1:1
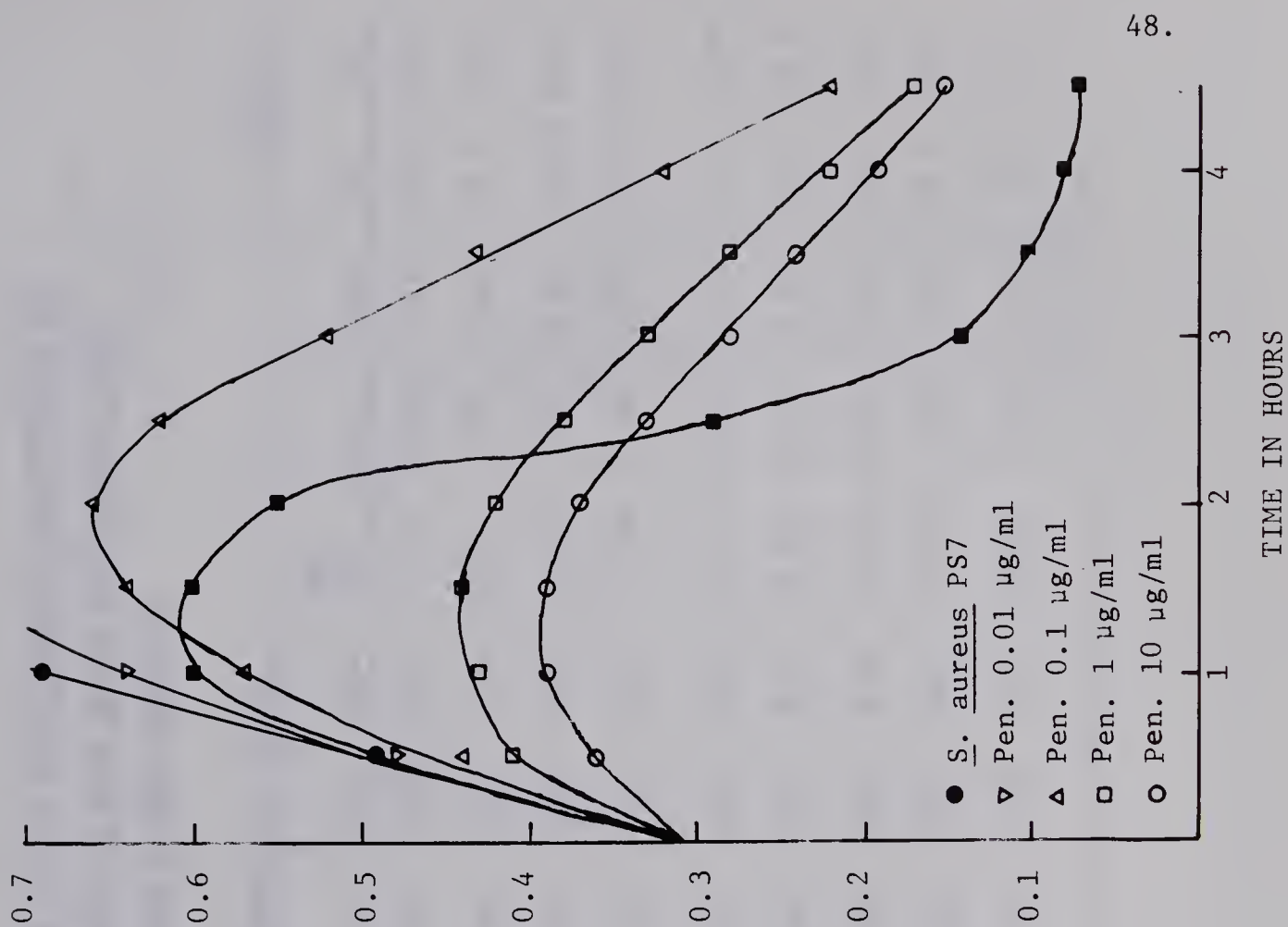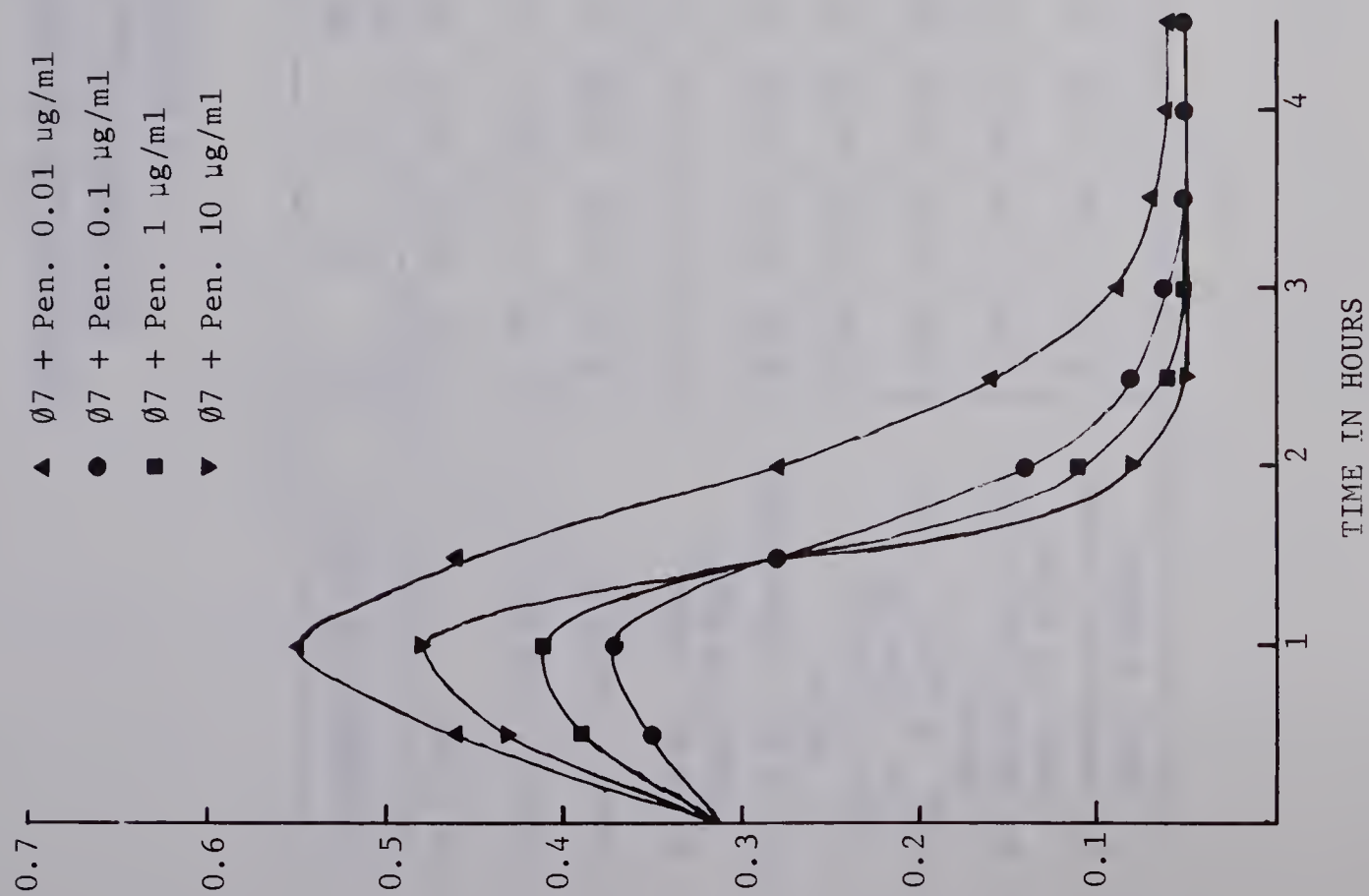
FIGURE 10. Effect of different concentrations of penicillin. Ø:cell, ca. 1:1.

48.

TABLE VI

COMPARISON OF THE EFFECTS DUE TO (a) SIMULTANEOUS ADDITION OF Ø7
AND PENICILLIN, (b) Ø7 ADDED ½ HOUR AFTER PENICILLIN, AND
(c) PENICILLIN ADDED ½ HOUR AFTER Ø7

| S. aureus PS7 plus: | 0 hr. | ½ hr. | 1 hr. | 1½ hrs. | 2 hrs. | 2½ hrs. | 3 hrs. | 3½ hrs. | 4 hrs. | Amax | ti | t½ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ABSORBANCE AT 600 mµ | | | | | | | | | | HOURS | |
| Ø7 alone | 0.30 | 0.50 | 0.64 | 0.72 | 0.69 | 0.59 | 0.43 | 0.22 | 0.11 | 0.72 | 3.3 | 3.8 |
| Pen., 10 µg/ml | 0.30 | 0.43 | 0.47 | 0.49 | 0.49 | 0.47 | 0.42 | 0.36 | 0.29 | 0.49 | 3.9 | (5.1) |
| Pen., 2 µg/ml | 0.30 | 0.46 | 0.50 | 0.53 | 0.52 | 0.50 | 0.46 | 0.38 | 0.33 | 0.53 | (4.2) | (4.9) |
| Ø7, ½ hr. after Pen., 10 µg/ml | 0.30 | 0.43 | 0.45 | 0.49 | 0.48 | 0.46 | 0.43 | 0.38 | 0.32 | 0.49 | (4.1) | (5.5) |
| Ø7, ½ hr. after Pen. 2 µg/ml | 0.30 | 0.47 | 0.49 | 0.53 | 0.51 | 0.48 | 0.43 | 0.37 | 0.28 | 0.53 | 3.9 | (4.8) |
| Pen., 10 µg/ml, ½ hr. after Ø7 | 0.30 | 0.51 | 0.57 | 0.54 | 0.42 | 0.23 | 0.11 | 0.06 | 0.02 | 0.57 | 2.3 | 2.8 |
| Pen., 2 µg/ml, ½ hr. after Ø7 | 0.30 | 0.47 | 0.56 | 0.53 | 0.40 | 0.24 | 0.04 | 0.05 | 0.02 | 0.56 | 2.3 | 2.8 |
| Ø7 immediately after Pen. 10 µg/ml | 0.30 | 0.40 | 0.41 | 0.41 | 0.21 | 0.07 | 0.03 | 0.02 | 0.01 | 0.41 | 1.8 | 2.3 |
| Ø7 immediately after Pen. 2 µg/ml | 0.30 | 0.43 | 0.45 | 0.43 | 0.22 | 0.08 | 0.03 | 0.02 | 0.01 | 0.45 | 1.8 | 2.3 |
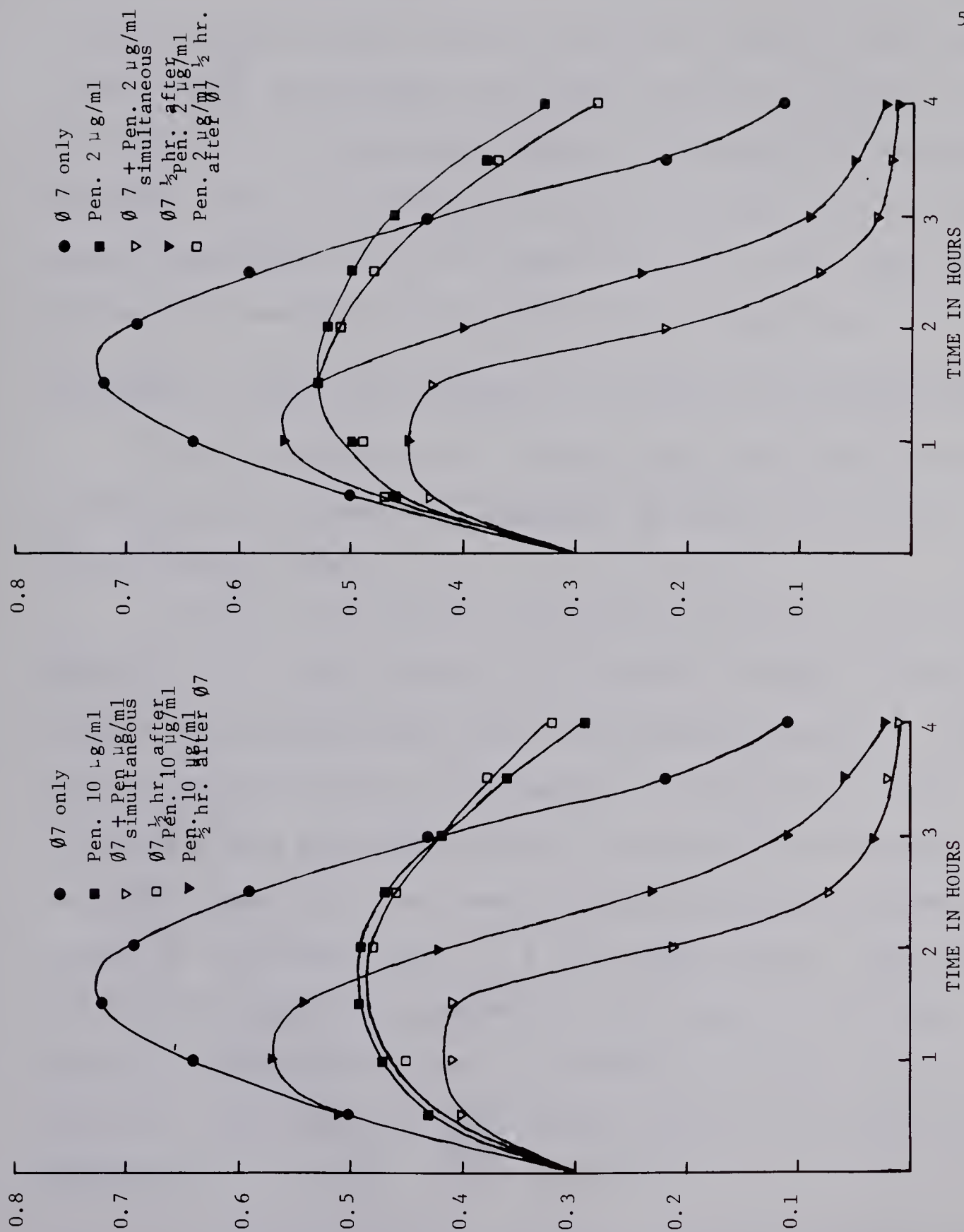
FIGURE 11. Comparison of the effect due to (a) simultaneous addition of Ø7 and penicillin; (b) Ø7 added ½ hour after penicillin; and (c) penicillin added ½ hour after Ø7. Ø:cell, ca. 2:1.

50.

resulted in an increase in the maximum absorbance as well as an increase in the half-lysis times.  The latter were still, however, less than those of Ø7 alone or of penicillin alone acting against S. aureus PS7.  The The results for 10 µg/ml penicillin and 2 µg/ml penicillin were almost identical.  When Ø7 was added half an hour after penicillin the results showed considerable delay in the onset and rate of rapid lysis, the curves here resembling those for the antibiotic used alone.

The effect of adding Ø7 at different intervals after penicillin (10 µg/ml)

It was decided to take a closer look at the effect of varying periods of pre-treatment of S. aureus PS7 by penicillin (10 µg/ml).  The Ø:cell ratio was 1.5:1.

Results are presented in Table VII and Fig. 12.  When Ø7 was added 2, 3, 4, 5, and 6 minutes after penicillin a slight tendency for delayed lysis was discernible;  the half-lysis times being 2.2, 2.3, 2.4, and 2.3 hours respectively, as compared to 2.0 hours for Ø7 added immediately after penicillin.  When Ø7 was added 10 minutes after penicillin, the delay in the onset and rate of rapid lysis became more evident as a half-lysis time of 3.1 hours would indicate.  This delay in the onset and rate of lysis became even more apparent as the time interval between penicillin and later addition of Ø7 was increased.  The half-lysis times for 20, 30 and 60 minutes being 4.2, 4.4, and 4.6 hours respectively.

The effect of penicillin (10 µg/ml) added at different intervals after Ø7

# TABLE VII

## THE EFFECT OF Ø7 ADDED AT DIFFERENT INTERVALS AFTER PENICILLIN (10 µg/ml)

| S. aureus PS7 plus: | ABSORBANCE AT 600 mµ | | | | | | | | | | | HOURS | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 hr. | ½ hr. | 1 hr. | 1½ hrs. | 2 hrs. | 2½ hrs. | 3 hrs. | 3½ hrs. | 4 hrs. | 4½ hrs. | Amax | ti | t½ |
| Ø7 added immediately after pen. | 0.31 | 0.37 | 0.38 | 0.31 | 0.16 | 0.09 | 0.07 | 0.06 | 0.06 | 0.06 | 0.38 | 1.5 | 2.0 |
| Ø7 2 min. after pen. | 0.31 | 0.38 | 0.38 | 0.33 | 0.19 | 0.10 | 0.06 | 0.06 | 0.06 | 0.06 | 0.38 | 1.6 | 2.2 |
| Ø7 3 min. after pen. | 0.31 | 0.38 | 0.38 | 0.33 | 0.21 | 0.13 | 0.08 | 0.07 | 0.06 | 0.06 | 0.38 | 1.5 | 2.3 |
| Ø7 4 min. after pen. | 0.31 | 0.37 | 0.37 | 0.34 | 0.23 | 0.14 | 0.09 | 0.07 | 0.06 | 0.06 | 0.37 | 1.4 | 2.4 |
| Ø7 5 min. after pen. | 0.31 | 0.39 | 0.38 | 0.35 | 0.24 | 0.14 | 0.10 | 0.07 | 0.07 | 0.06 | 0.39 | 1.7 | 2.4 |
| Ø7 6 min. after pen. | 0.31 | 0.39 | 0.38 | 0.35 | 0.22 | 0.13 | 0.09 | 0.07 | 0.06 | 0.06 | 0.39 | 1.7 | 2.3 |
| Ø7 10 min. after pen. | 0.31 | 0.38 | 0.38 | 0.37 | 0.30 | 0.22 | 0.16 | 0.11 | 0.08 | 0.08 | 0.38 | 1.9 | 3.1 |
| Ø7 20 min. after pen. | 0.31 | 0.39 | 0.39 | 0.39 | 0.37 | 0.33 | 0.27 | 0.21 | 0.17 | 0.14 | 0.39 | 2.7 | 4.2 |
| Ø7 30 min. after pen. | 0.31 | 0.39 | 0.39 | 0.39 | 0.38 | 0.34 | 0.28 | 0.22 | 0.18 | 0.15 | 0.39 | 2.8 | 4.4 |
| Ø7 60 min. after pen. | 0.31 | 0.39 | 0.39 | 0.39 | 0.38 | 0.35 | 0.30 | 0.24 | 0.19 | 0.16 | 0.39 | 2.9 | 4.6 |

Ø:cell ca. 1.5:1
Pen. 10 µg/ml

53.
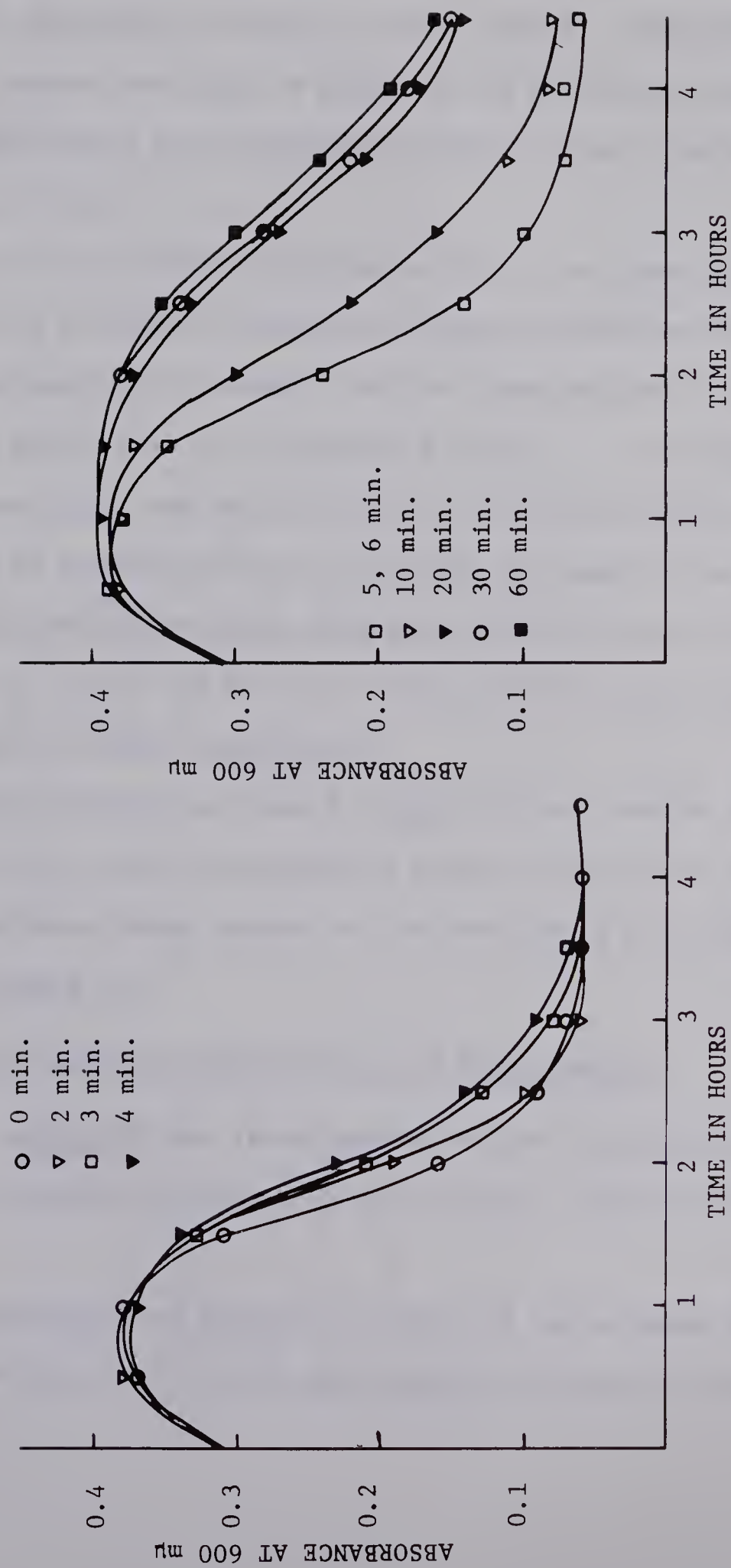


FIGURE 12.   Effect of adding Ø7 at different intervals after penicillin,(10 µg/ml).  Ø:cell, ca. 1.5:1

It was also decided to examine the responses resulting from the addition of penicillin at varying periods after Ø7. The penicillin concentration studied was again 10 µg/ml and the Ø:cell ratio was ca. 1.5:1.

The results are presented in Table VIII, and selected data are represented in Fig. 13.

As the time between addition of Ø7 and the later addition of penicillin was increased, there was a tendency towards an increase in the maximum absorbance attained. Parallel observations of the half-lysis times showed them to be slightly decreased. It can also be seen that when penicillin was added after Ø7 at different periods up to 30 minutes, in no instances was the half-lysis time greater than the value 1.8 hours for penicillin added immediately after Ø7, the $t\frac{1}{2}$ values for 2, 3, 4, 5, 6, 10, 20 and 30 minutes being 1.7, 1.7, 1.6, 1.6, 1.6, 1.4, 1.7, 1.6, and 1.8 hours respectively.

It is evident that once S. aureus PS7 was infected with Ø7, the later addition of penicillin caused no delay in the rate of rapid lysis, the lytic patterns being similar to that expected of Ø7 acting alone against S. aureus PS7.

The effect of different concentrations of D-cycloserine

S. aureus PS7 was tested against Ø7 and D-cycloserine in concentrations varying from 100 µg/ml to 0.1 µg/ml. The Ø:cell ratio was ca. 1.5:1.

The results are presented in Table IX and selected data are represented in Fig.14. It can be seen that when 100 µg/ml of D-cycloserine

## TABLE VIII

THE EFFECT OF PENICILLIN (10 μg/ml) ADDED AT DIFFERENT INTERVALS AFTER Ø7

| S. aureus PS7 plus: | ABSORBANCE AT 600 mμ | | | | | | | | HOURS | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | 0 hr. | ½ hr. | 1 hr. | 1½ hrs. | 2 hrs. | 2½ hrs. | 3 hrs. | Amax | ti | t½ |
| Pen. added immediately after Ø7 | 0.30 | 0.38 | 0.37 | 0.22 | 0.08 | 0.02 | 0.01 | 0.38 | 1.1 | 1.8 |
| Pen. added 2 min. after Ø7 | 0.30 | 0.38 | 0.38 | 0.22 | 0.07 | 0.03 | 0.01 | 0.38 | 1.1 | 1.7 |
| Pen. 3 min. after Ø7 | 0.30 | 0.38 | 0.39 | 0.22 | 0.05 | 0.01 | 0.01 | 0.39 | 1.2 | 1.7 |
| Pen. 4 min. after Ø7 | 0.30 | 0.39 | 0.40 | 0.21 | 0.05 | 0.01 | 0.01 | 0.40 | 1.2 | 1.6 |
| Pen. 5 min. after Ø7 | 0.30 | 0.40 | 0.42 | 0.21 | 0.05 | 0.02 | 0.01 | 0.42 | 1.2 | 1.6 |
| Pen. 6 min. after Ø7 | 0.30 | 0.39 | 0.38 | 0.10 | 0.05 | 0.02 | 0.01 | 0.39 | 1.1 | 1.4 |
| Pen. 10 min. after Ø7 | 0.30 | 0.40 | 0.44 | 0.26 | 0.04 | 0.03 | 0.01 | 0.44 | 1.4 | 1.7 |
| Pen. 20 min. after Ø7 | 0.30 | 0.42 | 0.46 | 0.28 | 0.05 | 0.02 | 0.01 | 0.46 | 1.5 | 1.6 |
| Pen. 30 min. after Ø7 | 0.30 | 0.45 | 0.50 | 0.36 | 0.07 | 0.03 | 0.01 | 0.50 | 1.6 | 1.8 |
| Pen. 60 min. after Ø7 | 0.30 | 0.48 | 0.53 | 0.44 | 0.28 | 0.10 | 0.05 | 0.53 | 1.9 | 2.4 |

Ø:cell ca. 1.5:1
Pen. 10 μg/ml
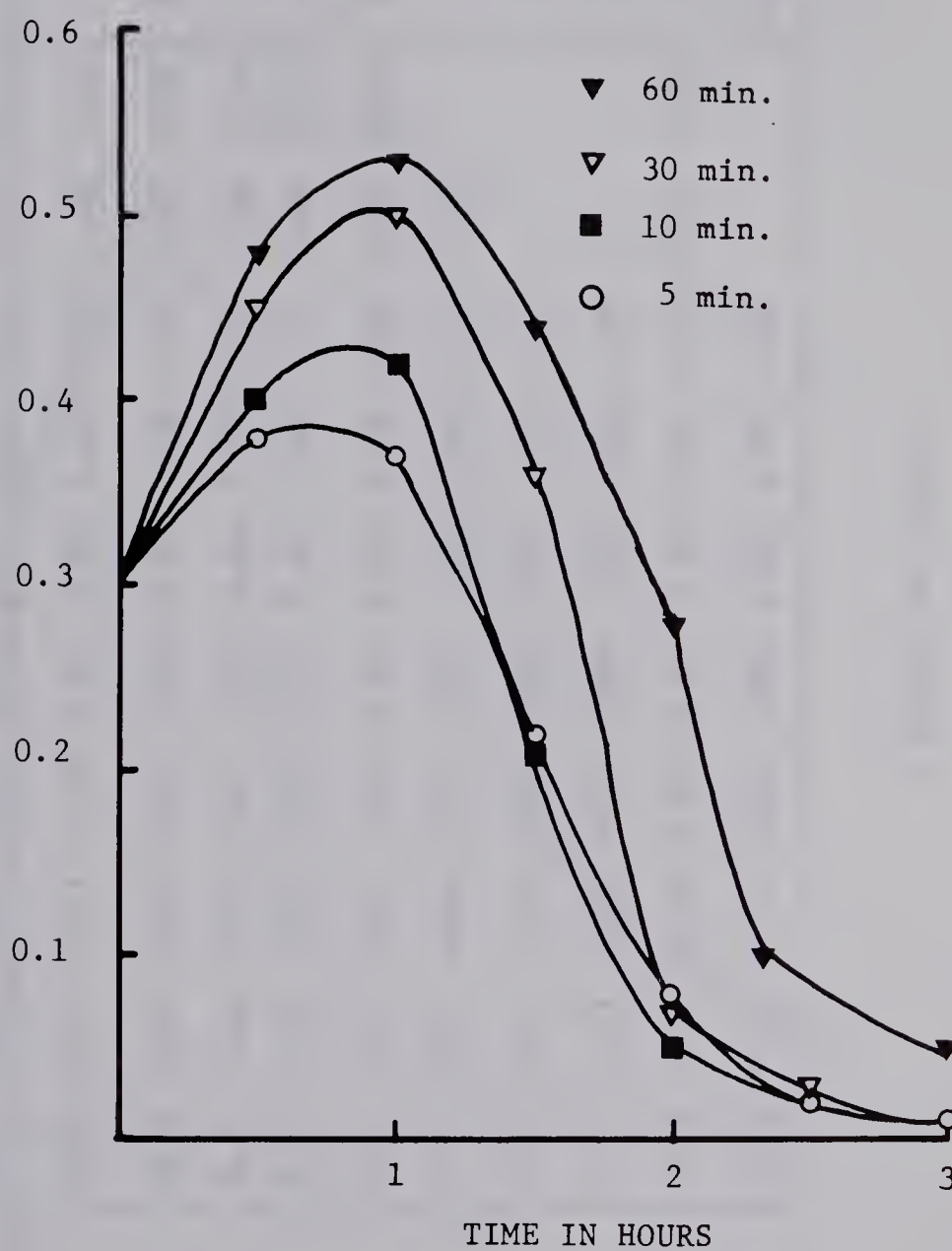
FIGURE 13.  Effect of penicillin (10 μg/ml) added at
different intervals after Ø7.  Ø:cell, ca. 1.5:1

TABLE IX

THE EFFECT OF DIFFERENT CONCENTRATIONS OF D-CYCLOSERINE

| S. aureus PS7 plus: | ABSORBANCE AT 600 mμ | | | | | | | | | | | HOURS | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 hr. | ½ hr. | 1 hrs. | 1½ hrs. | 2 hrs. | 2½ hrs. | 3 hrs. | 3½ hrs. | 4 hrs. | 4½ hrs. | Amax | ti | t½ |
| D-cyclos. 100 μg/ml + ø7 | 0.31 | 0.43 | 0.47 | 0.19 | 0.04 | 0.03 | 0.02 | 0.02 | 0.01 | 0.01 | 0.47 | 1.3 | 1.6 |
| D-cyclos. 10 μg/ml + ø7 | 0.31 | 0.45 | 0.59 | 0.59 | 0.48 | 0.35 | 0.16 | 0.10 | 0.07 | 0.05 | 0.59 | 2.6 | 3.1 |
| D-cyclos. 1 μg/ml + ø7 | 0.31 | 0.46 | 0.63 | 0.62 | 0.58 | 0.39 | 0.18 | 0.11 | 0.09 | 0.09 | 0.63 | 2.7 | 3.2 |
| D-cyclos. 0.1 μg/ml | 0.31 | 0.48 | 0.65 | 0.64 | 0.59 | 0.40 | 0.18 | 0.12 | 0.09 | 0.07 | 0.65 | 2.7 | 3.3 |
| D-cyclos. 100 μg/ml | 0.31 | 0.43 | 0.51 | 0.55 | 0.54 | 0.51 | 0.49 | 0.48 | 0.45 | 0.42 | 0.55 | (6.6) | (9.5) |
| D-cyclos. 10 μg/ml | 0.31 | 0.50 | 0.69 | 0.85 | 0.98 | 1.10 | 1.16 | 1.22 | 1.30 | - | >1.30 | - | - |
| D-cyclos. 1 μg/ml | 0.31 | 0.51 | 0.70 | 0.85 | 1.00 | 1.10 | 1.22 | 1.30 | - | - | >1.30 | - | - |
| D-cyclos. 0.1 μg/ml | 0.31 | 0.51 | 0.70 | 0.87 | 1.00 | 1.16 | 1.22 | 1.30 | - | - | >1.30 | - | - |
| ø7 only | 0.31 | 0.50 | 0.66 | 0.65 | 0.54 | 0.36 | 0.16 | 0.10 | 0.08 | 0.06 | 0.66 | 2.6 | 3.1 |
| S. aureus PS7 only | 0.31 | 0.51 | 0.71 | 0.85 | 1.00 | 1.16 | 1.22 | 1.30 | - | - | >1.30 | - | - |

ø:cell ca. 1.5:1
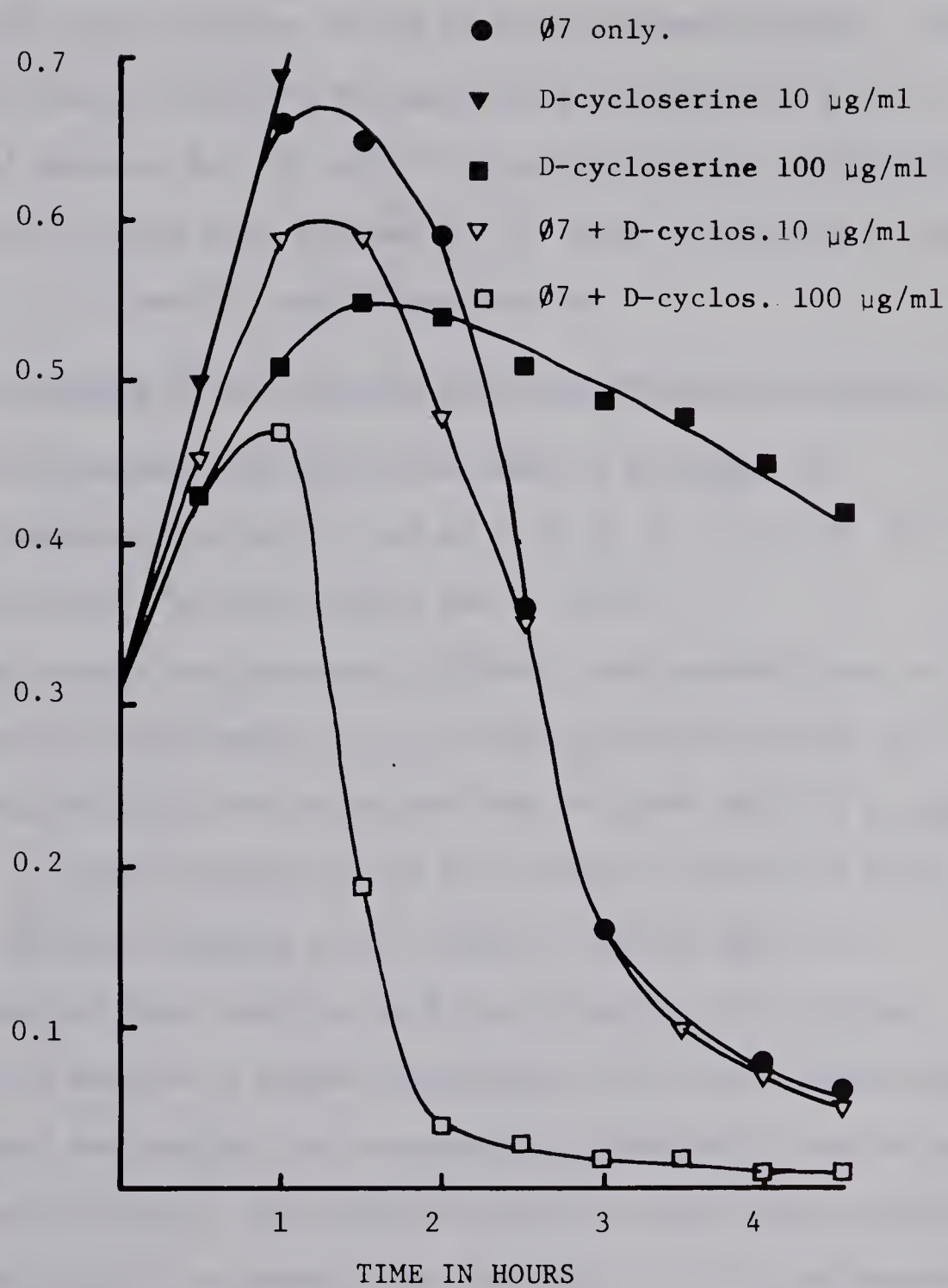
FIGURE 14.   The effect of different concentrations of
            D-cycloserine.   Ø:cell ca. 1.5:1.

was used there was a decrease in the maximum absorbance values. Also,

a half-lysis time of 1.6 hours was observed as compared to 3.1 and 9.5

hours for Ø7 alone,or for 100 µg/ml D-cycloserine alone, respectively.

Similar lytic patterns were obtained for Ø7 alone, as well as in the

presence of 10, 1, and 0.1 µg/ml D-cycloserine.

## The effect of adding Ø7 at different intervals after D-cycloserine (100 µg/ml)

D-cycloserine (100 µg/ml) was added to S. aureus PS7

suspension immediately after Ø7, and at 2, 3, 4, 5, 6, 10, 20, 30, and

60 minute periods. The Ø:cell ratio was ca. 1.5:1.

The results are presented in Table X and selected data in Fig. 15.

The addition of D-cycloserine up to 10 minutes prior to Ø7 did not appear

to affect significantly the extent and rate of rapid lysis of S. aureus

PS7 by Ø7. A slight increase in the half-lysis time from 1.4 to 1.8

hours was, however, observed as the interval between addition of

D-cycloserine and later addition of Ø7 was extended to 10 minutes. At

20, 30, and 60 minutes, a marked curtailment of the rate and the extent

of rapid lysis was evident, the corresponding half-lysis times being

5.9, 6.9, and 9.1 hours. The lytic patterns at these times resembled

more and more that to be expected for 100 µg/ml of D-cycloserine acting

alone against S. aureus PS7.

## The effect of different concentrations of vancomycin

S. aureus PS7 was tested against Ø7 in combination with

vancomycin at concentrations of 10, 5, and 1 µg/ml. The Ø:cell ratio was

1.5:1.

# TABLE X

## THE EFFECT OF ADDING Ø7 AT DIFFERENT INTERVALS AFTER D-CYLOSERINE (100µg/ml)

| S. aureus PS7 plus: | ABSORBANCE AT 600 mµ | | | | | | | | | | Amax | HOURS | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 hr. | ½ hr. | 1 hr. | 1½ hrs. | 2 hrs. | 2½ hrs. | 3 hrs. | 3½ hrs. | 4 hrs. | 4½ hrs. | | ti | t½ |
| Ø7 immediately after D-cyclos. | 0.30 | 0.37 | 0.37 | 0.17 | 0.03 | 0.02 | 0.02 | 0.02 | 0.02 | 0.01 | 0.37 | 1.2 | 1.4 |
| Ø7 2 min. after D-cyclos. | 0.30 | 0.38 | 0.37 | 0.16 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.01 | 0.38 | 1.2 | 1.4 |
| Ø7 3 min. after D-cyclos. | 0.30 | 0.38 | 0.38 | 0.16 | 0.03 | 0.03 | 0.02 | 0.02 | 0.02 | 0.02 | 0.38 | 1.2 | 1.4 |
| Ø7 4 min. after D-cyclos. | 0.30 | 0.38 | 0.39 | 0.18 | 0.03 | 0.03 | 0.03 | 0.02 | 0.02 | 0.02 | 0.39 | 1.2 | 1.4 |
| Ø7 5 min. after D-cyclos. | 0.30 | 0.38 | 0.38 | 0.17 | 0.03 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.38 | 1.2 | 1.6 |
| Ø7 6 min. after D-cyclos. | 0.30 | 0.38 | 0.38 | 0.19 | 0.03 | 0.03 | 0.03 | 0.03 | 0.02 | 0.02 | 0.38 | 1.2 | 1.6 |
| Ø7 10 min. after D-cyclos. | 0.30 | 0.38 | 0.39 | 0.28 | 0.09 | 0.05 | 0.04 | 0.03 | 0.03 | 0.02 | 0.39 | 1.5 | 1.8 |
| Ø7 20 min. after D-cyclos. | 0.30 | 0.38 | 0.40 | 0.40 | 0.36 | 0.33 | 0.30 | 0.28 | 0.25 | 0.23 | 0.40 | 3.1 | (5.9) |
| Ø7 30 min. after D-cyclos. | 0.30 | 0.38 | 0.41 | 0.42 | 0.41 | 0.40 | 0.39 | 0.37 | 0.35 | 0.33 | 0.42 | (5.2) | (6.9) |
| Ø7 60 min. after D-cyclos. | 0.30 | 0.39 | 0.41 | 0.43 | 0.42 | 0.39 | 0.38 | 0.36 | 0.36 | 0.34 | 0.43 | (5.2) | (9.1) |

Ø:cell ca. 1.5:1

D-cyclos. 100 µg/ml

60.
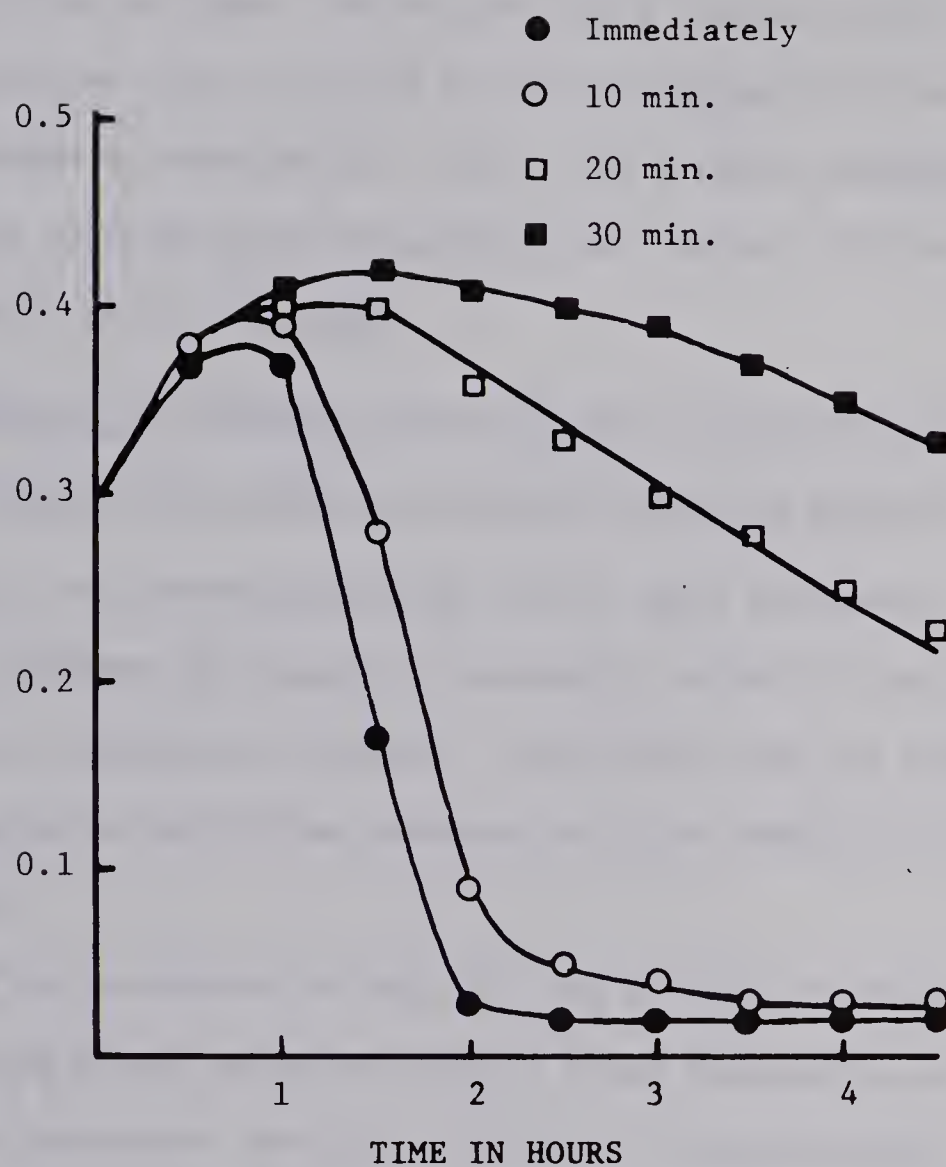
FIGURE 15. The effect of adding Ø7 at different intervals after
D-cycloserine (100 μg/ml). Ø:cell, ca. 1.5:1.

The results are presented in Table XI and partly in Fig. 16, and show that 10 µg/ml of vancomycin added together with Ø7 caused considerable inhibition of lysis, the pattern being similar to that observed when vancomycin alone was used atainst S. aureus PS7. The smaller concentrations of vancomycin, 5 µg/ml, and 1 µg/ml, permitted rapid lysis. These concentrations of antibiotics, however, did not significantly inhibit bacterial growth.

The effect of adding Ø7 at different intervals after vancomycin (10 µg/ml) and of adding vancomycin (10 µg/ml) at different intervals after Ø7.

S. aureus PS7 was tested against Ø7 and 10 µg/ml vancomycin. The effect of the addition of vancomycin immediately after Ø7, and at 5, 10, and 30 minute periods was followed. The effect when the order of addition of vancomycin and Ø7 was reversed was also examined for the same time intervals.

The results are presented in Table XII and in Fig. 17, where it can be seen that when Ø7 was added immediately after vancomycin and for increasing periods thereafter, the rate of lysis was significantly inhibited. When vancomycin was added immediately after Ø7 there was also inhibition of lysis of S. aureus PS7 suspensions. With greater delay in the addition of vancomycin, however, there was decreased inhibition, and rapid lysis ensued, the half-lysis times for the 5, 10, and 15 minute periods being 2.4, 1.9, and 2.1 hours respectively.

The effect of different concentrations of bacitracin.

The effect of Ø7 in combination with 100, 10, and 1 µg/ml of

## TABLE XI

### THE EFFECT OF DIFFERENT CONCENTRATIONS OF VANCOMYCIN

| S. aureus PS7 plus: | ABSORBANCE AT 600 mμ | | | | | | | | | | Amax | HOURS | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | 0 hr. | ½ hr. | 1 hr. | 1½ hrs. | 2 hrs. | 2½ hrs. | 3 hrs. | 3½ hrs. | 4 hrs. | 4½ hrs. | | ti | t½ |
| Van., 10 μg/ml + Ø7 | 0.31 | 0.42 | 0.43 | 0.43 | 0.40 | 0.38 | 0.37 | 0.34 | 0.33 | 0.31 | 0.43 | 4.3 | (9.4) |
| Van., 5 μg/ml + Ø7 | 0.31 | 0.47 | 0.63 | 0.59 | 0.52 | 0.41 | 0.18 | 0.10 | 0.08 | 0.07 | 0.63 | 2.7 | 3.2 |
| Van., 1 μg/ml + Ø7 | 0.31 | 0.47 | 0.63 | 0.59 | 0.52 | 0.43 | 0.20 | 0.12 | 0.09 | 0.07 | 0.63 | 2.8 | 3.4 |
| Van., 10 μg/ml | 0.31 | 0.43 | 0.45 | 0.45 | 0.44 | 0.43 | 0.42 | 0.40 | 0.39 | 0.38 | 0.45 | 7.0 | (14.6) |
| Van., 5 μg/ml | 0.31 | 0.50 | 0.69 | 0.83 | 0.96 | 1.10 | - | - | - | - | >1.10 | - | - |
| Van., 1 μg/ml | 0.31 | 0.50 | 0.69 | 0.83 | 0.96 | 1.10 | - | - | - | - | >1.10 | - | - |
| Ø7 only | 0.31 | 0.50 | 0.64 | 0.59 | 0.55 | 0.38 | 0.16 | 0.10 | 0.08 | 0.07 | 0.64 | 2.6 | 3.2 |
| S. aureus PS7 only | 0.31 | 0.53 | 0.72 | 0.89 | 1.02 | 1.19 | - | - | - | - | >1.19 | - | - |

Ø:cell, ca. 1.5:1

FIGURE 16. The effect of different concentrations of vancomycin. Ø:cell, ca. 1.5:1.

## TABLE XII

### THE EFFECT OF ADDING Ø7 AT DIFFERENT INTERVALS AFTER VANCOMYCIN (10 µg/ml) AND OF ADDING VANCOMYCIN (10 µg/ml) AT DIFFERENT INTERVALS AFTER Ø7

| S. aureus PS7 plus: | ABSORBANCE AT 600 mµ | | | | | | | | | | | HOURS | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | 0 hr. | ½ hr. | 1 hr. | 1½ hrs. | 2 hrs. | 2½ hrs. | 3 hrs. | 3½ hrs. | 4 hrs. | 4½ hrs. | Amax | ti | t½ |
| Ø7 only | 0.31 | 0.50 | 0.60 | 0.59 | 0.48 | 0.25 | 0.10 | 0.06 | 0.05 | 0.04 | 0.60 | 2.3 | 2.7 |
| Van. | 0.31 | 0.41 | 0.42 | 0.41 | 0.39 | 0.38 | 0.36 | 0.36 | 0.35 | 0.34 | 0.42 | (6.0) | – |
| Ø7 immediately after van. | 0.31 | 0.40 | 0.41 | 0.37 | 0.31 | 0.28 | 0.26 | 0.24 | 0.24 | 0.21 | 0.41 | 2.0 | (6.2) |
| Ø7 5 min. after van. | 0.31 | 0.40 | 0.41 | 0.41 | 0.37 | 0.33 | 0.32 | 0.31 | 0.31 | 0.28 | 0.41 | 4.0 | – |
| Ø7 10 min. after van. | 0.31 | 0.40 | 0.40 | 0.41 | 0.38 | 0.37 | 0.35 | 0.34 | 0.34 | 0.32 | 0.41 | 4.5 | – |
| Ø7 30 min. after van. | 0.31 | 0.40 | 0.41 | 0.41 | 0.39 | 0.38 | 0.37 | 0.36 | 0.36 | 0.34 | 0.41 | (6.0) | – |
| Van. immediately after Ø7 | 0.31 | 0.39 | 0.41 | 0.36 | 0.29 | 0.25 | 0.23 | 0.21 | 0.20 | 0.18 | 0.41 | 1.9 | – |
| Van. 5 min. after Ø7 | 0.31 | 0.44 | 0.46 | 0.34 | 0.19 | 0.15 | 0.12 | 0.11 | 0.09 | 0.08 | 0.46 | 1.6 | 2.4 |
| Van. 10 min. after Ø7 | 0.31 | 0.45 | 0.46 | 0.31 | 0.14 | 0.09 | 0.07 | 0.06 | 0.05 | 0.04 | 0.46 | 1.5 | 1.9 |
| Van. 30 min. after Ø7 | 0.31 | 0.51 | 0.53 | 0.40 | 0.19 | 0.08 | 0.04 | 0.03 | 0.03 | 0.03 | 0.53 | 1.7 | 2.1 |

Ø: cell ca. 1.5:1
Van. 10 µg/ml
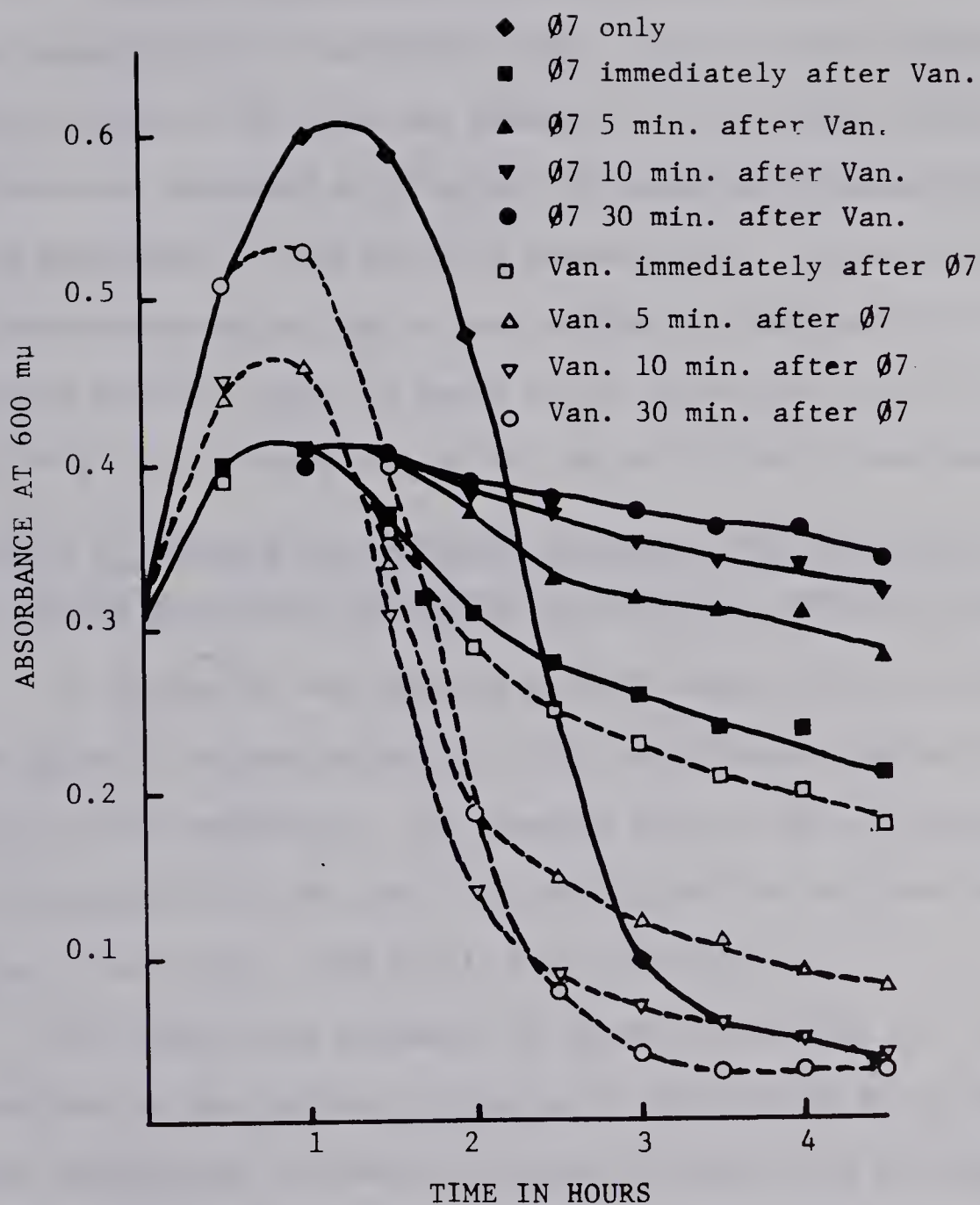
FIGURE 17. The effect of adding Ø7 at different intervals after vancomycin (10 μg/ml) and of adding vancomycin (10 μg/ml), at different intervals after Ø7. Ø:cell, ca. 1.5:1.

bacitracin acting against S. aureus PS7 was examined. The Ø:cell ratio

was 1.5:1. Results are presented in Table XIII and Fig. 18. With the

lowest concentration of bacitracin used, 1 µg/ml, a lytic pattern

similar to that for Ø7 alone was observed. As the concentration of

bacitracin was increased to 10 µg and 100 µg/ml the maximum absorbances

reached decreased to 0.68 and 0.52 respectively. The rate of rapid

lysis also decreased, as can be seen in Fig. 18, although the half-lysis

times were similar, being 3.0 hours for Ø7 alone, and 3.0, 3.0, and 3.1

hours for Ø7 in combination with 100, 10 and 1 µg/ml of bacitracin.

## The effect of adding Ø7 at different intervals after bacitracin (100 µg/ml) and of adding bacitracin (100 µg/ml) at different intervals after Ø7

S. aureus PS7 was infected with Ø7 immediately after addition

of 100 µg/ml of bacitracin and at 5, 10, and 30 minute periods after the

addition of the antibiotic. The reversed order of Ø7 and bacitracin was

also investigated for the same time period, and for the same concen-

tration of bacitracin. The Ø:cell ratio was ca. 1:1.

The results are presented in Table XIV and Fig. 19. It can

be seen that as the interval following the addition of Ø7 to bacitracin-

treated cultures was increased, the rate of rapid lysis decreased. The

half-lysis times for Ø7 immediately after bacitracin and at 5, 10, and

30 minute intervals were 2.1, 2.7, 3.5 and 4.5 hours respectively, the

curves resembling more and more that for 100 µg/ml bacitracin alone

acting against S. aureus PS7. When bacitracin was added after Ø7, the

rate of lysis was rapid. The maximum absorbance values when bacitracin

TABLE XIII

THE EFFECT OF DIFFERENT CONCENTRATIONS OF BACITRACIN

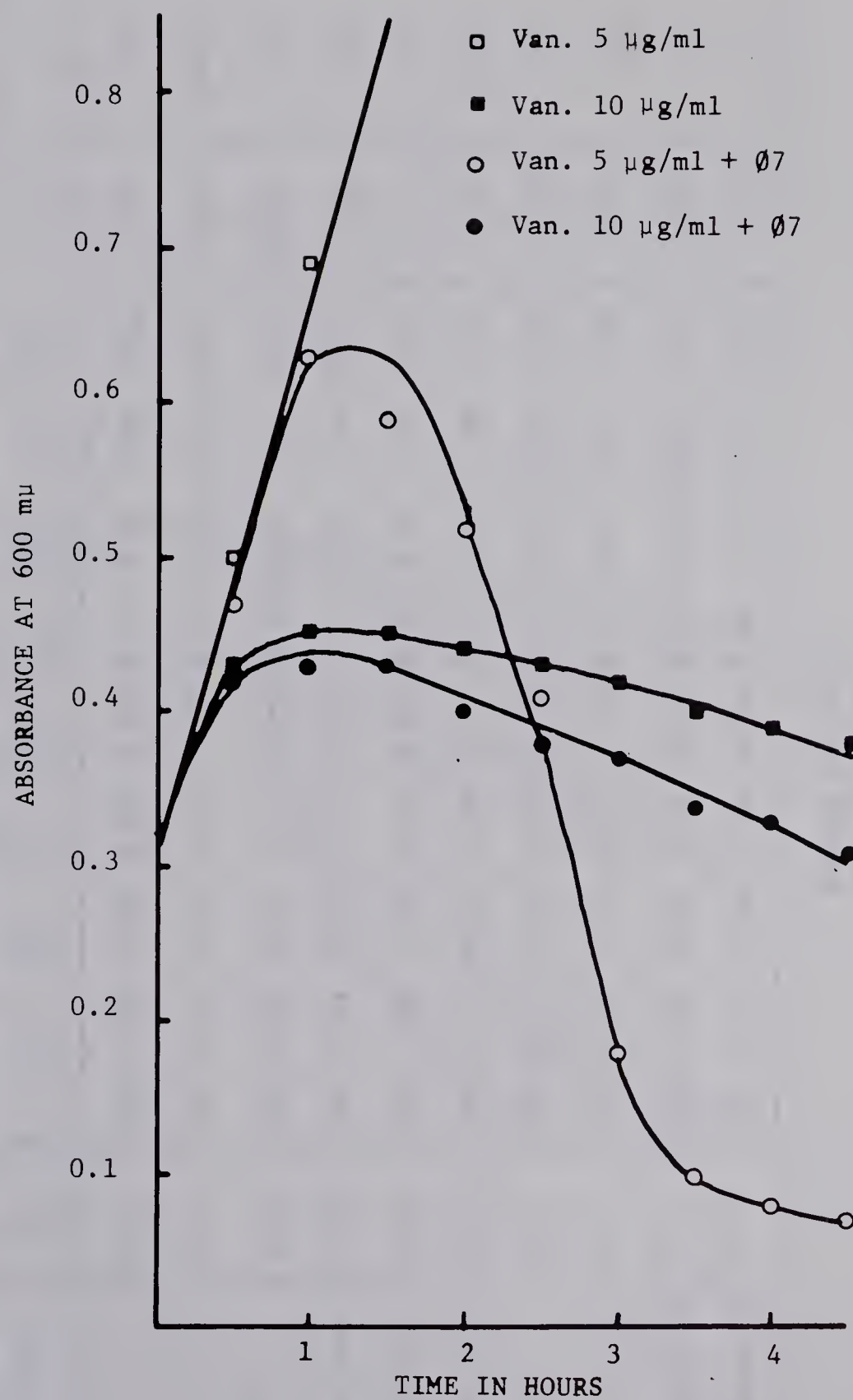| S. aureus PS7 plus: | ABSORBANCE AT 600mµ | | | | | | | | | | | HOURS | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 hr. | ½ hr. | 1 hr. | 1½ hr. | 2 hrs. | 2½ hrs. | 3 hrs. | 3½ hrs. | 4 hrs. | 4½ hrs. | Amax | ti | ti |
| Bacit. 100 µg/ml + Ø7 | 0.31 | 0.41 | 0.41 | 0.36 | 0.28 | 0.20 | 0.15 | 0.11 | 0.08 | 0.07 | 0.41 | 1.8 | 3.0 |
| Bacit. 10 µg/ml + Ø7 | 0.31 | 0.46 | 0.52 | 0.51 | 0.40 | 0.28 | 0.14 | 0.07 | 0.05 | 0.04 | 0.52 | 2.3 | 3.0 |
| Bacit. 1 µg/ml + Ø7 | 0.31 | 0.48 | 0.65 | 0.68 | 0.67 | 0.47 | 0.18 | 0.11 | 0.08 | 0.08 | 0.68 | 2.8 | 3.1 |
| Bacit. 100 µg/ml | 0.31 | 0.43 | 0.42 | 0.41 | 0.39 | 0.36 | 0.33 | 0.31 | 0.27 | 0.23 | 0.43 | 3.4 | (6.5) |
| Bacit. 10 µg/ml | 0.31 | 0.48 | 0.59 | 0.66 | 0.75 | 0.78 | 0.83 | 0.89 | 0.92 | 1.10 | >1.10 | - | - |
| Bacit. 1 µg/ml | 0.31 | 0.52 | 0.70 | 0.85 | 1.10 | 1.13 | 1.22 | 1.35 | 1.40 | - | >1.40 | - | - |
| Ø7 only added | 0.31 | 0.52 | 0.64 | 0.63 | 0.59 | 0.28 | 0.14 | 0.10 | 0.08 | 0.07 | 0.64 | 2.4 | 3.0 |
| S. aureus only | 0.31 | 0.55 | 0.75 | 0.89 | 1.05 | 1.19 | 1.26 | 1.35 | 1.40 | - | >1.40 | - | - |
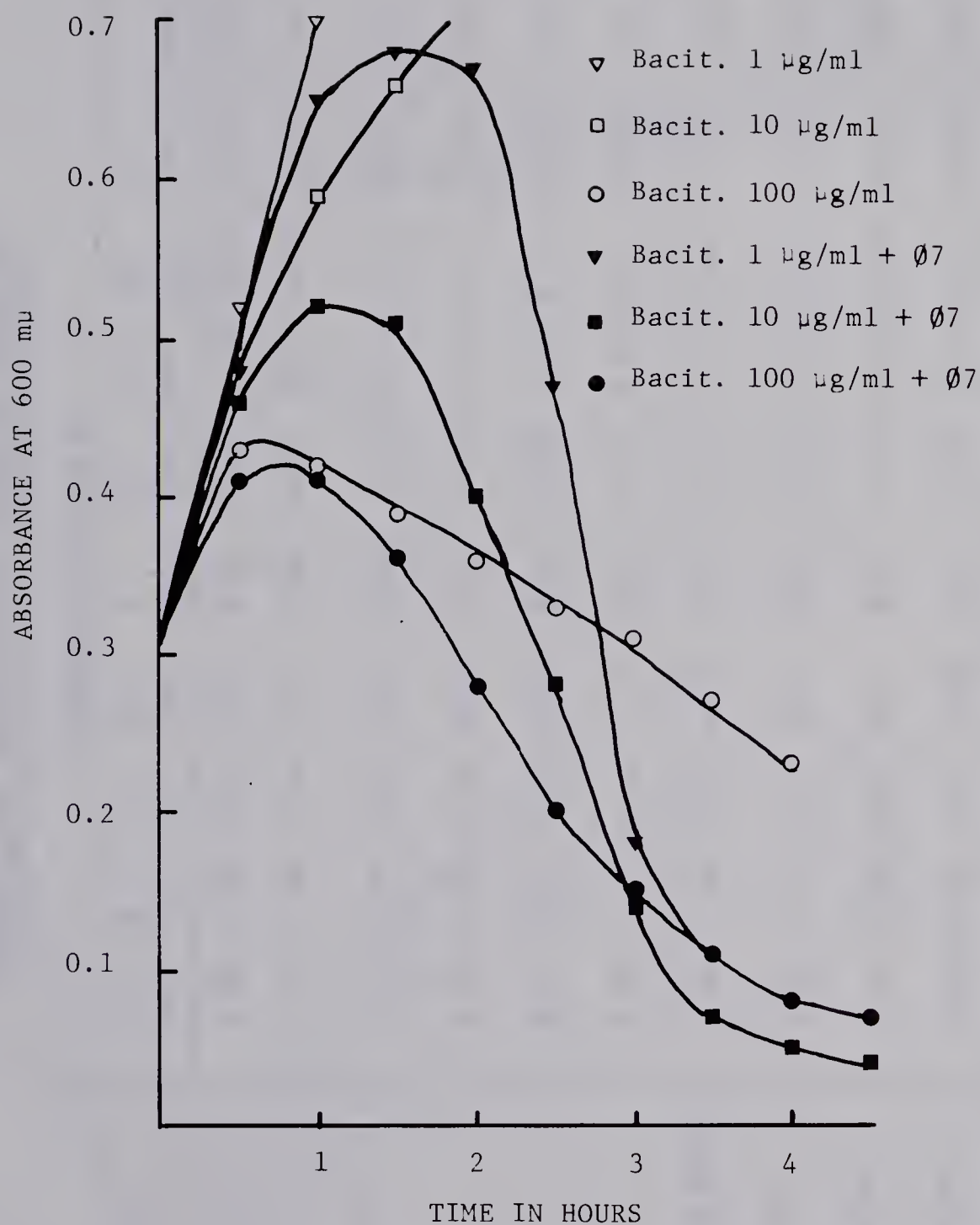
Ø:cell ca. 1.5:1

FIGURE 18. The effect of different concentrations of bacitracin. Ø:cell, ca. 1.5:1

TABLE XIV

THE EFFECT OF ADDING Ø7 AT DIFFERENT INTERVALS AFTER BACITRACIN (100 µg/ml) AND OF ADDING BACITRACIN (100 µg/ml) AT DIFFERENT INTERVALS AFTER Ø7

| S. aureus PS7 plus: | ABSORBANCE AT 600 mµ | | | | | | | | | | HOURS | |
| | 0 hr. | ½ hr. | 1 hr. | 1½ hrs. | 2 hrs. | 2½ hrs. | 3 hrs. | 3½ hrs. | 4 hrs. | Amax | ti | t½ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ø7 only added | 0.30 | 0.46 | 0.61 | 0.65 | 0.63 | 0.46 | 0.22 | 0.13 | 0.10 | 0.65 | 2.7 | 3.3 |
| Bacit. | 0.30 | 0.40 | 0.42 | 0.41 | 0.39 | 0.35 | 0.31 | 0.26 | 0.21 | 0.42 | 3.0 | 4.7 |
| Ø7 immediately after Bacit. | 0.30 | 0.38 | 0.40 | 0.34 | 0.20 | 0.06 | 0.03 | 0.02 | 0.02 | 0.40 | 1.6 | 2.1 |
| Ø7, 5 min. after Bacit. | 0.30 | 0.38 | 0.40 | 0.37 | 0.30 | 0.18 | 0.10 | 0.05 | 0.03 | 0.40 | 2.0 | 2.7 |
| Ø7, 10 min. after Bacit. | 0.30 | 0.39 | 0.41 | 0.40 | 0.35 | 0.30 | 0.22 | 0.15 | 0.10 | 0.41 | 2.4 | 3.5 |
| Ø7, 30 min. after Bacit. | 0.30 | 0.39 | 0.41 | 0.41 | 0.38 | 0.34 | 0.30 | 0.25 | 0.20 | 0.41 | 2.9 | 4.5 |
| Bacit. immediately after Ø7 | 0.30 | 0.38 | 0.40 | 0.33 | 0.15 | 0.03 | 0.02 | 0.02 | 0.02 | 0.40 | 1.6 | 2.0 |
| Bacit., 5 min. after Ø7 | 0.30 | 0.40 | 0.43 | 0.34 | 0.06 | 0.02 | 0.02 | 0.02 | 0.02 | 0.43 | 1.5 | 1.7 |
| Bacit., 10 min. after Ø7 | 0.30 | 0.42 | 0.45 | 0.37 | 0.04 | 0.02 | 0.02 | 0.02 | 0.02 | 0.45 | 1.5 | 1.6 |
| Bacit., 30 min. after Ø7 | 0.30 | 0.48 | 0.56 | 0.40 | 0.11 | 0.03 | 0.02 | 0.02 | 0.02 | 0.56 | 1.6 | 1.9 |

Ø:cell ca. 1:1
Bacit. 100 µg/ml
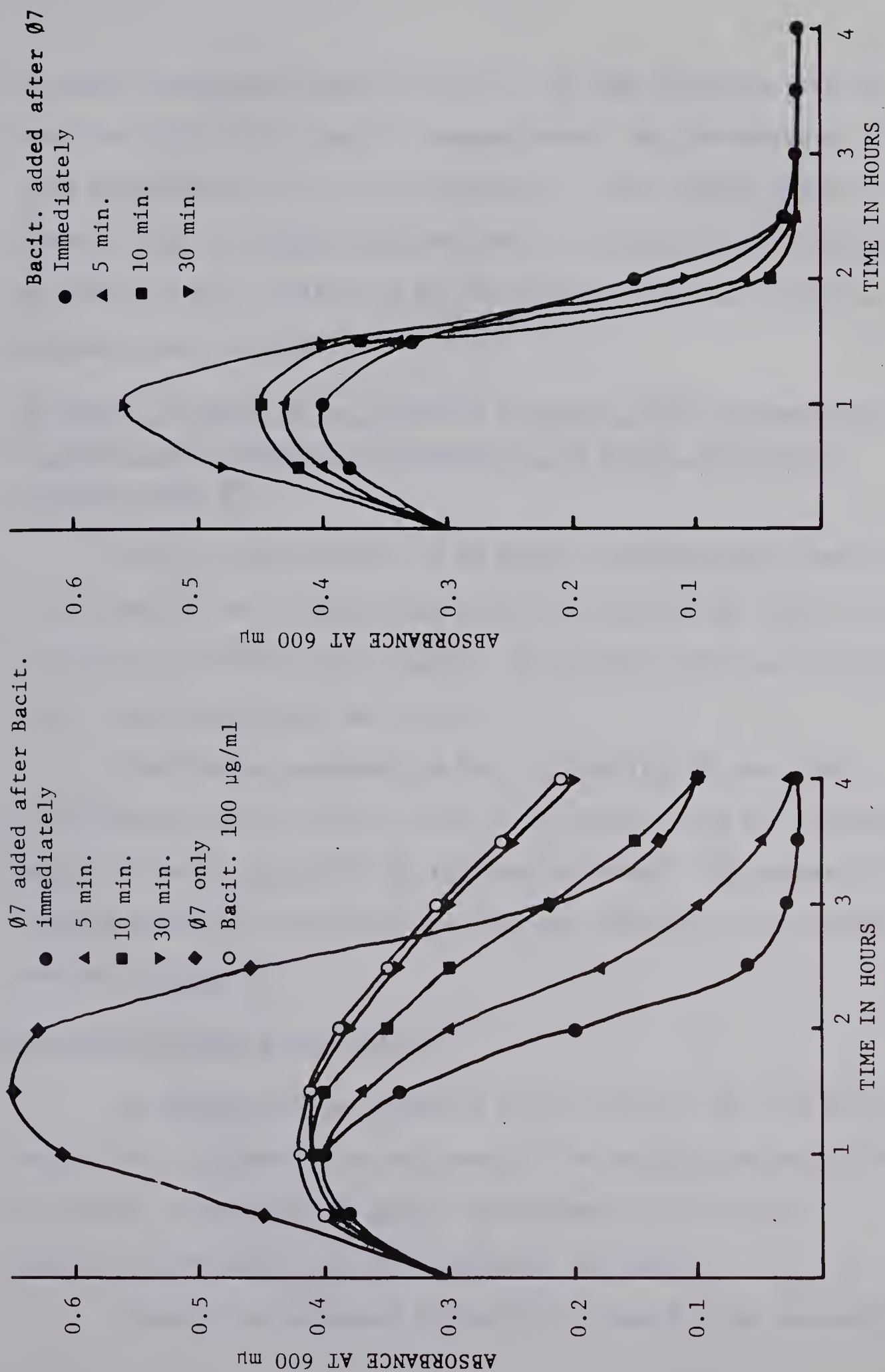
FIGURE 19. The effect of adding Ø7 at different intervals after bacitracin (100 μg/ml), and of adding bacitracin(100 μg/ml) at different intervals after Ø7. Ø:cell, ca. 1:1.

was added immediately after Ø7 and at 5, 10, and 30 minute periods,
were 0.40, 0.43, 0.45, and 0.56 respectively. The corresponding half-
lysis times were 2.0, 1.7, 1.6, 1.9 hours. These values, and the
curves in Fig. 19 suggest that the rate of rapid lysis was increased as
the period between addition of Ø7 and the later addition of 100 μg of
bacitracin was increased.

The effect of adding Ø7 at different intervals after chloramphenicol
(50 μg/ml) and of adding chloramphenicol (50 μg/ml) at different
intervals after Ø7.

Using a concentration of 50 μg/ml chloramphenicol, the effect
of the addition of Ø7 immediately after this antibiotic, and at 5, 10,
and 30 minute intervals was studied. The reverse order was also investi-
gated. Phage:cell ratio was ca. 1:1.

The results presented in Table XV and Fig. 20 show that
chloramphenicol given before, or up to 30 minutes after Ø7, abolished
rapid lysis of S. aureus PS7 by this bacteriophage. The pattern of
changing absorbances resembled that for the antibiotic alone acting
against S. aureus PS7.

The effect of high Ø:cell ratio.

S. aureus PS7 was subjected to the action of Ø7 at a Ø:cell
ratio of 6:1, in combination with each of the antibiotics penicillin,
(10 μg/ml), vancomycin (10 μg/ml), D-cycloserine (100 μg/ml),
bacitracin (100 μg/ml) and chloramphenicol (20 μg/ml).

Results are presented in Table XVI. Rapid lysis occurred in

TABLE XV

THE EFFECT OF ADDING Ø7 AT DIFFERENT INTERVALS AFTER CHLORAMPHENICOL (50 μg/ml) AND OF ADDING CHLORAMPHENICOL (50 μg/ml) AT DIFFERENT INTERVALS AFTER Ø7

| S. aureus PS7 plus: | ABSORBANCE AT 600 mμ | | | | | | | | Amax | HOURS | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 hr. | ½ hr. | 1 hr. | 1½ hrs. | 2 hr. | 2½ hrs. | 3 hrs. | 3½ hrs. | | ti | t½ |
| Ø7 only added | 0.31 | 0.45 | 0.61 | 0.65 | 0.63 | 0.42 | 0.13 | 0.08 | – | 2.6 | 2.9 |
| Chloramph. | 0.31 | 0.40 | 0.46 | 0.48 | 0.50 | 0.52 | 0.54 | 0.55 | – | – | – |
| Ø7 immediately after chloramph. | 0.31 | 0.40 | 0.44 | 0.47 | 0.49 | 0.52 | 0.55 | 0.55 | – | – | – |
| Ø7 5 min. after chloramph. | 0.31 | 0.39 | 0.44 | 0.46 | 0.47 | 0.50 | 0.52 | 0.54 | – | – | – |
| Ø7 10 min. after chloramph. | 0.31 | 0.40 | 0.43 | 0.46 | 0.48 | 0.50 | 0.53 | 0.54 | – | – | – |
| Ø7 30 min. after chloramph. | 0.31 | 0.40 | 0.43 | 0.47 | 0.50 | 0.51 | 0.54 | 0.55 | – | – | – |
| Chloramph. immediately after Ø7 | 0.31 | 0.40 | 0.42 | 0.45 | 0.48 | 0.50 | 0.53 | 0.54 | – | – | – |
| Chloramph. 5 min. after Ø7 | 0.31 | 0.39 | 0.42 | 0.45 | 0.48 | 0.50 | 0.52 | 0.53 | – | – | – |
| Chloramph. 10 min. after Ø7 | 0.31 | 0.39 | 0.42 | 0.44 | 0.47 | 0.49 | 0.51 | 0.52 | – | – | – |
| Chloramph. 30 min. after Ø7 | 0.31 | 0.45 | 0.46 | 0.50 | 0.53 | 0.54 | 0.58 | 0.59 | – | – | – |

Ø:cell, ca. 1:1
(Chloramph. 50 μg/ml)
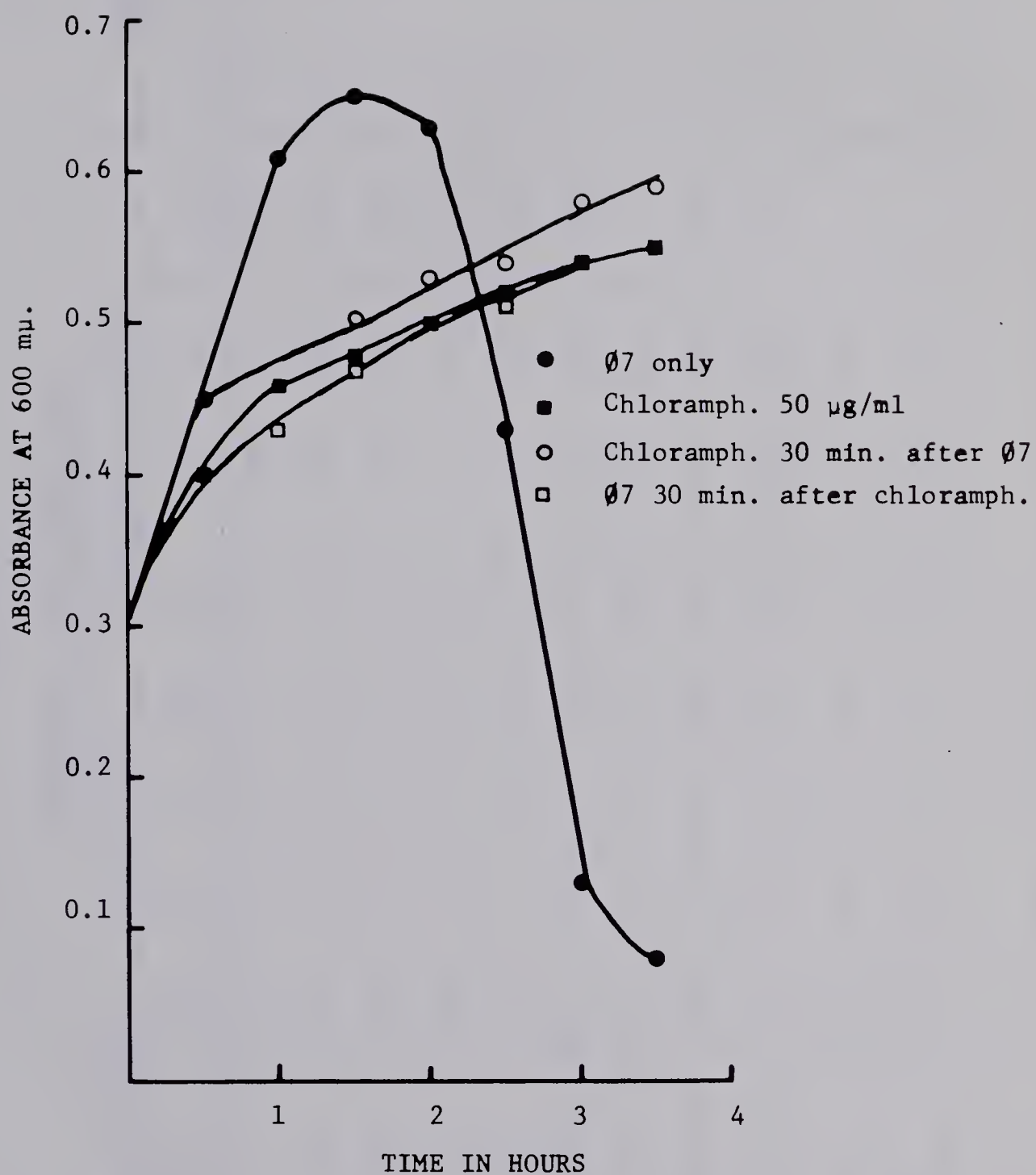
FIGURE 20.  The effect of adding Ø7 at different intervals after
chloramphenicol (50 µg/ml), and of adding chloramphenicol
(50 µg/ml) at different intervals after Ø7.

75.

TABLE XVI

THE EFFECT OF HIGH Ø:CELL RATIO

| S. aureus PS7 plus: | ABSORBANCE AT 600 mµ | | | | | | | | HOURS | |
| | 0 hr. | ½ hr. | 1 hr. | 1½ hrs. | 2 hrs. | 2½ hrs. | 3 hrs. | Amax | ti | t½ |
|---|---|---|---|---|---|---|---|---|---|---|
| Ø7 only | 0.31 | 0.42 | 0.50 | 0.43 | 0.22 | 0.10 | 0.08 | 0.50 | 1.8 | 2.2 |
| * Pen. 10 µg/ml + Ø7 | 0.31 | 0.34 | 0.34 | 0.20 | 0.06 | 0.02 | 0.01 | 0.34 | 1.2 | 1.7 |
| ‡ Pen. 10 µg/ml + Ø7 | 0.31 | 0.34 | 0.35 | o.21 | 0.06 | 0.03 | 0.02 | 0.35 | 1.2 | 1.7 |
| * Van. 10 µg/ml + Ø7 | 0.31 | 0.37 | 0.38 | 0.32 | 0.19 | 0.11 | 0.09 | 0.38 | 1.5 | 2.4 |
| ‡ Van. 10 µg/ml + Ø7 | 0.31 | 0.37 | 0.39 | 0.34 | 0.22 | 0.14 | 0.10 | 0.39 | 1.5 | 2.3 |
| * D-cyclos. 100 µg/ml + Ø7 | 0.31 | 0.36 | 0.37 | 0.26 | 0.13 | 0.07 | 0.05 | 0.36 | 1.4 | 1.9 |
| ‡ D-cyclos.100 µg/ml + Ø7 | 0.31 | 0.37 | 0.37 | 0.27 | 0.11 | 0.06 | 0.05 | 0.37 | 1.4 | 1.9 |
| * Bacit. 100 µg/ml + Ø7 | 0.31 | 0.38 | 0.35 | 0.27 | 0.14 | 0.04 | 0.03 | 0.38 | 1.4 | 2.0 |
| ‡ Bacit. 100 µg/ml + Ø7 | 0.31 | 0.38 | 0.36 | 0.29 | 0.15 | 0.04 | 0.02 | 0.38 | 1.4 | 2.0 |
| * Chloramphen. 20 µg/ml + Ø7 | 0.31 | 0.41 | 0.46 | 0.50 | 0.52 | 0.53 | 0.55 | - | - | - |
| ‡ Chloramphen. 20 µg/ml + Ø7 | 0.31 | 0.41 | 0.46 | 0.48 | 0.51 | 0.52 | 0.55 | - | - | - |

Ø:cell ca. 6:1

* Ø7 added first and antibiotic immediately after~.
‡ Antibiotic added first and Ø7 immediately after.

all systems except that for chloramphenicol, where a reduction in
absorbance was not observed.

# DISCUSSION OF IN VITRO INVESTIGATIONS.

At the outset of this investigation it was found that $\emptyset7$ could be propagated on S. aureus PS7 in ordinary nutrient broth. It soon became evident, however, that under such conditions the rate of lysis of S. aureus PS7 by $\emptyset7$ was rather slow; in addition, early experiments showed that there was little adsorption of $\emptyset7$ to S. aureus PS7 in 15 minutes. In view of the findings of Rountree (75) that phages 7, 42B and 42E had a specific requirement of 50 $\mu$g/ml $CaCl_2$ for adsorption, it was considered desirable to investigate the effect of added $CaCl_2$. That this might be important was also suggested by analogy with the results of Delbruck (11) who showed that a mutant of coliphage T4 required $Ca^{++}$ for adsorption. Also Adams (1) had reported that coliphage T5 needed $Ca^{++}$ for development and replication although it did not require this cation for adsorption. Blair and Williams (9) reported their observations at the Staphylococcus Reference Laboratory, Colindale, London, which indicated that even slight excess heating of media may bind calcium in such a manner that the ionic form is not available for the more $Ca^{++}$ dependent phages. It seems likely that this latter explanation could account for the slow adsorption and lysis observed in the $\emptyset7$ - S. aureus PS7 system when the broth medium was not supplemented with $CaCl_2$.

When $CaCl_2$ was added to the nutrient broth, rapid lysis of S. aureus PS7 by $\emptyset7$ was obtained. It was found however, that beyond

75 µg/ml added $CaCl_2$, there was no further increase in the efficiency of lysis, indicating that above this level the adsorption of Ø7 to S. aureus PS7 was no longer concentration-dependent. The use of 100 µg/ml of $CaCl_2$ to supplement nutrient broth in later experiments gave con-sistent lytic and adsorption responses.

The adsorption of Ø7 to log phase S. aureus PS7 suspensions in $NBCa^{++}$ was found to be rapid. In 5 minutes ca. 92 - 95% of added Ø7 was adsorbed. The adsorption constant over this period was $2.17 \times 10^{-9}$ ml $min^{-1}$, a value about 10 times that obtained by Krueger (42) for Staphy-lococcus aureus and a staphylococcus phage. Krueger (42) also reported that the adsorption constant was the same for live and heat-killed bacteria. Delbruck (11) however, showed that the adsorption rate constants for coliphages to Escherichia coli varied considerably depending on the physiological state of the bacteria; maximum adsorption rates being obtained when bacteria were grown with aeration and were in the log phase. The high adsorption rate of Ø7 to S. aureus PS7 as compared with Krueger's reported results may have been due more to differences in the physiological state of the bacteria than to differences in the phage and bacterial strains used.

The results of the adsorption of Ø7 to S. aureus PS7 also showed that initially the bulk of the Ø adsorbed rapidly and the later fractions more slowly. This is in keeping with the observations of Schlesinger (77), who showed that any batch of Ø-lysate was a hetero-geneous mixture which could be divided into 3 main fractions - a large

fraction which adsorbed rapidly, a smaller fraction which adsorbed more slowly and a very small residual fraction which did not adsorb at all.

The presence of the antibiotics penicillin, D-cycloserine, oxacillin, chloramphenicol, vancomycin, and bacitracin did not affect the adsorption of $\emptyset$7 to S. aureus PS7. Of these antibiotics penicillin (10), vancomycin (69), and chloramphenicol (95) are known to bind rapidly to bacterial cells. Bacitracin, which competes with penicillin for binding sites (69) and oxacillin, which is closely related to penicillin, are also expected to be rapidly bound. The binding of penicillin (10) and vancomycin (69) is irreversible, while that of chloramphenicol is known to be reversible on washing (95). Since for the concentration of anti-biotics used there are expected to be several thousands of antibiotic molecules available to each bacterial cell in the adsorption mixture, and inasmuch as there was no interference with the rate of adsorption of $\emptyset$7, it is reasonable to conclude that the binding of the antibiotics used to S. aureus PS7 does not take place at the $\emptyset$ receptor sites.

One-step growth of $\emptyset$7 with S. aureus PS7 as host at 37$^{\circ}$C showed a latent period of ca. 30 minutes, a burst size of ca. 95, and a rise period of ca. 22 minutes. Other experiments showed that 120 minutes after infection, titrable $\emptyset$ had increased 140-fold. In the presence of 10 $\mu$g/ml of penicillin, 100 $\mu$g/ml D-cycloserine, and 10 $\mu$g/ml of vancomycin there was only a 2, 3, and 5-fold increase respectively in titrable $\emptyset$ after 120 minutes. Assays for a comparable period in the presence of 100 $\mu$g/ml of bacitracin and 20 $\mu$g/ml of chloramphenicol

showed a decrease of ca. 50% in the titrable Ø compared to input.  Since

the adsorption of Ø7 to bacteria was unaffected by antibiotic and

effectively greater than 90% complete in 5 minutes, the plaques obtained

from bacteria initially infected would seem to suggest a certain degree

of reversibility of the effects of the antibiotic permitting plaque

formation when the samples were diluted for assay.  This is in keeping

with the known reversibility of the binding of chloramphenicol on

washing (95), but is not in accord with the irreversible nature of the

binding of vancomycin to bacterial cells.  Irreversibility of binding of

antibiotic does not however necessarily rule out reversibility of some

antibiotic effects, an observation certainly true for penicillin, as

reported by Rogers (17).

A common denominator in the activity of the antibiotics penicillin,

vancomycin, bacitracin, and D-cycloserine is that they affect murein

synthesis.  On the basis of this consideration alone, it would be

expected that the cell wall of affected bacteria should be structurally

weakened, thus facilitating lysis by phage-induced lysozymes when Ø and

antibiotics were permitted to act in conjunction.  However, the situation

is not as simple as this.

The action of penicillin and D-cycloserine has been shown to be

almost exclusively directed against mechanisms involved in cell wall

synthesis (69).  It would be expected, therefore, that these two

antibiotics would more closely resemble each other in their influence on

the activity of Ø7 against S. aureus PS7.  Results appear to bear this

out.  Both antibiotics permitted a limited increase in $\emptyset$ yield.  When  they were added prior to $\emptyset$ infection the extent of the inhibition of rapid lysis appeared to be comparable for up to 10 minutes; beyond this period the penicillin-treated systems showed a greater decrease in turbidity than was the case for D-cycloserine.

When added simultaneously with $\emptyset 7$, penicillin or D-cycloserine did not prevent lysis of S. aureus PS7.  On the contrary, lysis was hastened.  It would seem therefore that while there is a considerable reduction in the amount of complete $\emptyset$ produced, there appears to be no interference with the production or activity of $\emptyset$-directed lysozymes. When either of these antibiotics was added after infection there was no inhibition of rapid lysis, again indicating no interference with lysozyme activity.

In contrast to the effects observed with the two above-mentioned antibiotics, there was marked inhibition of lysis when vancomycin in a concentration inhibitory to bacterial growth, was added to S. aureus PS7 simultaneously with $\emptyset 7$.  As expected, therefore, pre-treatment with vancomycin also caused marked inhibition of rapid lysis.  However, where the addition of vancomycin to previously $\emptyset$-infected bacteria was delayed, the effect of the antibiotic decreased as the time of delayed addition was increased.  These observations would seem to indicate that vancomycin interferes with early $\emptyset$-directed activity.

Reynolds (70) has pointed out that bacitracin and vancomycin appear to be similar in most of their effects on sensitive bacteria.

Results using the ∅7 - S. aureus PS7 system appear to reflect such

similarity. There was inhibition of rapid lysis when bacitracin was

added at the same time as ∅7, although this was not so clearly manifested

as with vancomycin. The two antibiotics were also similar in that on

delayed addition to ∅7-infected bacteria the inhibition of rapid lysis

was decreased proportionally to the delay. However, bacitracin and

vancomycin were dissimilar in that while the latter appeared to allow

some increase in titrable ∅, the former did not.

The results obtained for the effect of penicillin, D-cycloserine,

vancomycin and bacitracin on the lysis of S. aureus PS7 by ∅7 contrasted

sharply with those obtained with chloramphenicol. Using this latter

antibiotic, addition either together with ∅7, or before ∅7, or up to 30

minutes after ∅7, caused virtually complete inhibition of rapid lysis.

Where the antibiotic was added either at the same time as ∅7 or before ∅7

the observed inhibition of lysis could easily be explained on the basis

of the action of the antibiotic in preventing ∅ replication (88) due to

inhibition of the synthesis of early enzymes and other enzymes required

for ∅ replication, including lysozymes. Added 30 minutes after ∅7, it

might be expected that the formation of lysozymes (which commences midway

in the latent period (88)) would already be completed and, as a result,

there would be some readily observable decrease in the turbidity of the

suspensions. Traut and Munro (91) found that chloramphenicol inhibits

the effect of puromycin in releasing phenylananine peptides from poly-

phenylalanyl-s-RNA attached to ribosomes. It is interesting to speculate

that Ø-induced lysozymes may still be attached to ribosomes towards the end of the latent period of the Ø-replication cycle and that the addition of chloramphenicol as late as 30 minutes after infection may in some way have prevented the release of the lytic enzymes from the ribosomes, with the result that lysis and accompanying decrease in the turbidity was not observed.

The results of infection with high concentration of Ø7 would appear to underscore some of the observations already indicated. As expected, penicillin and D-cycloserine did not prevent rapid lysis of the bacteria. Vancomycin, however, did not inhibit lysis in the presence of high Ø concentration (Ø:cell, ca. 6:1) as it did for a lower Ø concentration (Ø:cell; ca. 1.5:1). "Lysis from without" would not satisfactorily explain the decreased turbidity observed in the case of penicillin, D-cycloserine, bacitracin, and vancomycin-treated suspensions of S. aureus PS7, since the turbidity of chloramphenicol-treated suspensions in the presence of high Ø7 concentration did not show a similar decrease.

# III

## STAPHYLOCOCCUS AUREUS, STAPHYLOPHAGE 7

## AND ANTIBIOTICS IN MICE

## INTRAPERITONEAL INOCULATION OF MICE
## WITH <u>STAPHYLOCOCCUS</u> <u>AUREUS</u> PS7

In order to obtain information relating to the lethality of <u>S</u>. <u>aureus</u> PS7 in mice, an <u>in vivo</u> experiment employing the stair-step design of Finney (21) was performed. In this and subsequent experiments involving mice, treatments were randomly assigned, in keeping with the principles outlined by Steel and Torrie (87).

## Virulence of <u>S</u>. <u>aureus</u> PS7 for mice

One ml of <u>S</u>. <u>aureus</u> PS7 suspension of absorbance 1.0, harvested from an overnight culture on nutrient agar, was used to inoculate 100 ml of NB $Ca^{++}$. The inoculated culture in a 250 ml conical flask was inoculated at $37^{o}C$ in a water-bath with shaking until the absorbance was ca. 0.5. This log phase suspension was concentrated 20:1 by centrifugation. Serial tenfold dilutions of the concentrated suspension were made using NB $Ca^{++}$ as diluent.

Mice (17.9 gm average weight) were intraperitoneally inoculated with 0.2 ml of the concentrated suspension and 0.2 ml of each dilution using a $2\frac{1}{2}$ ml hypodermic syringe and a 26G $\frac{1}{2}$inch needle. One mouse was used at each dilution. Death after 24 hours was noted and the observations were continued for 3 days.

The results showed that only the mouse receiving the highest dose ($1.93 \times 10^9$ cfu) <u>S</u>. <u>aureus</u> PS7 died within 24 hours.

LD$_{50}$ determination.

A suspension of S. aureus PS7 containing 1.83 x 10$^{11}$ cfu/ml was prepared. Serial tenfold dilutions of the concentrated suspension in NB Ca$^{++}$ were made and 0.2 ml portions of each dilution were inoculated intraperitoneally into 5 male mice of average weight 17.7 gm.

The results at 24 hours indicated that S. aureus PS7 had a low order of virulence for mice; only 3 out of 5 animals died at the highest cell dose (10$^{10.56}$ cfu) used. This lack of virulence may have been the result of repeated propagation on artificial media; an observation in keeping with those of Koenig and Melly (41). An attempt was therefore made to increase the virulence of the staphylococcal strain by repeated passage in the peritoneal cavity of mice, as suggested by Van de Velde (93).

Attempt to increase the virulence of S. aureus PS7 for mice.

Using one of the mice which had received an inoculum of 10$^{10.56}$ cfu from the LD$_{50}$ experiment described above, 2.5 ml of NB Ca$^{++}$ was intro- duced into the peritoneal cavity ca. 30 hours post-inoculation. The animal was then anaesthetized and 1.5 ml of fluid withdrawn from the peritoneum and introduced intraperitoneally into another mouse (mouse #2). At the end of 24 hours this mouse was also anaesthetized, peritoneal fluid withdrawn and portions used to make smears on glass slides. A sample was also spread on a blood agar plate.

Under the microscope, the smears showed occasional intracellular and extracellular Gram-positive cocci. Only 12 colonies of S. aureus PS7

grew, after overnight incubation at 37°C, on the blood agar plate.

A suspension of the 12 staphylococcal colonies was made in ca.
1 ml NB Ca$^{++}$ and used to inoculate a third mouse. After 6 hours,
peritoneal fluid from mouse #3 was passaged to yet another mouse (mouse
#4), and this process was repeated at 6-hour intervals for a total of 4
passages. Peritoneal fluid from the last mouse in the series (mouse #7)
was smeared on glass slides, and inoculated on nutrient agar plates.
Incubation of the nutrient agar plates at 37°C overnight yielded only 13
colonies. A pooled suspension of these colonies in ca. 1 ml NB Ca$^{++}$ was
made and a portion used to inoculate ca. 50 ml of NB Ca$^{++}$. After
incubation to an absorbance of ca. 0.45, a twenty-fold concentration of
the cultured organisms was made.

The virulence of this suspension was investigated by intraperi-
toneal inoculation of 0.2 ml portions of the concentrate and its 1/10
dilution in NB Ca$^{++}$; 2 mice were used for each suspension.

No deaths were observed after 24 hours, indicating that the
organism had not acquired increased virulence for mice as a result of
successive intraperitoneal transfer.

## SELECTION OF A MOUSE-VIRULENT STRAIN OF STAPHYLOCOCCUS.

As a result of the above failure to increase the virulence of S. aureus PS7, it became necessary to look for a more lethal strain of staphylococcus.

A number of S. aureus cultures were obtained from the phage-typing section of this laboratory and from the collection of Dr. J. I. Payne. A loopful of each culture was used to inoculate 5 ml of NB $Ca^{++}$ in 16 mm x 120 mm test tubes and grown to an absorbance of ca. 1.0. Nutrient agar plates were inoculated from each culture and the organisms tested for antibiotic sensitivity by the disc-plate method, except for D-cycloserine where a cup-plate technique was employed.

The results of these tests are presented in Table XVII. Cultures 4, 18, and 19 showed sensitivity patterns similar to that for S. aureus PS7. The sensitivity tests of cycloserine were ambiguous. Cultures 4, 18, 19, together with S. aureus PS7 were re-tested using a tube technique which indicated sensitivity of all four strains to a cycloserine concentration of 100 μg/ml in NB $Ca^{++}$, as revealed by absence of visible turbidity after overnight incubation at 37°C.

### Virulence of selected staphylococcal strains for mice

The virulence of S. aureus strains 4, 18, and 19 was tested by intraperitoneal inoculation of 0.2 ml of appropriate suspensions of absorbance ca. 1.0 into each of 3 male mice.

One death resulted from each of strains 4 and 18, and two deaths from strain 19.

TABLE XVII

ANTIBIOTIC SENSITIVITY OF S. AUREUS STRAINS

| Antibiotic | S. aureus cultures | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | PS7 |
| Vancomycin 30 µg | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Novobiocin 5 µg | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Chloramphenicol 5 µg | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Bacitracin 2 µg | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Streptomycin 2 µg | + | + | + | + | + | + | + | + | + | + | + | + | + | + | 0 | + | + | + | 0 | 0 | + |
| Penicillin 1 µg | + | + | + | + | + | + | + | + | + | + | 0 | 0 | + | + | + | + | + | + | + | + | + |
| Oxacillin 2 µg | + | + | + | + | + | + | + | + | + | + | ± | 0 | + | + | + | + | + | + | + | + | + |
| Ampicillin 5 µg | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| D-cycloserine, 100 µg/ml | ? | ? | ? | ± | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ± | ± | ? |
| *D-cycloserine, 100 µg/ml | | | | + | | | | | | | | | | | | | | | | | + |

+ = sensitive
± = slightly sensitive
0 = not sensitive
? = ambiguous

* Retested by tube method.

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Plaque-forming ability of selected staphylococcal strains.

Nutrient agar plates were seeded with samples of a broth culture of each staphylococcal strain and after drying, serial dilutions of stock Ø7 were spotted on each bacterial lawn with a loop as outlined by Blair and Williams (9). After overnight incubation, the "routine test dilution" (RTD) (9), and plaque-forming ability was noted for each strain. S. aureus PS7 was included for comparison. Results are recorded below.

| S. aureus strain | RTD | Plaques |
|:---:|:---:|:---:|
| 4 | $10^{-4}$ | + |
| 18 | $10^{-4}$ | + |
| 19 | $10^{-4}$ | + |
| PS7 | $10^{-4}$ | + |

(+ = plaques formed)

Efficiency of plating (EOP) of Ø7 on selected staphylococcal strains, relative to S. aureus PS7.

Stock Ø7 suspension was assayed, using each staphylococcal strain as the seed culture.

Results showed S. aureus 19 gave the highest EOP value (83.2%) relative to S. aureus PS7.

On the basis of these observations S. aureus 19 was selected for further study since it showed the following desirable properties:

1. Wide range of sensitivity to antibiotics, comparable to S. aureus PS7.
2. Virulence for mice via the intraperitoneal route.

3.   Allows Ø7 to form plaques with high EOP relative to S. aureus PS7.

## Determination of $LD_{50}$ for S. aureus 19

S. aureus 19 from an overnight nutrient agar plate was harvested using NB $Ca^{++}$ as diluent, and the suspension adjusted to an absorbance of ca. 1.0.  A 1% inoculum of this suspension in NB $Ca^{++}$ was grown to an absorbance of ca. 0.6, at $37^{o}C$.  This suspension was concentrated 20:1 by centrifugation, and the concentrated organisms and serial tenfold dilutions in NB $Ca^{++}$ were used to inoculate 0.2 ml portions intraperitoneally into each of 5 mice (average weight 27 gm).

The results of this investigation gave an $LD_{50}$ of 2.9 x $10^7$ cfu, as determined by the procedure of Reed and Meunch (69).

## LETHALITY OF ANTIBIOTICS FOR MICE, AND
## OTHER CONTROL EXPERIMENTS.

Because of reports by Hamre et al. (30), Donovich and Rake (14),
Farrar, Kent and Elliott (20) and Kelly and Silverman (39), that some
antibiotics are lethal for mice, it was decided to determine to what extent
this was true under the present experimental conditions.

### Lethality of antibiotics for mice

Antibiotics were injected intraperitoneally into mice.  Each
antibiotic was contained in 0.2 ml of an aqueous solution, except in the
case of chloramphenicol, where 0.5 ml and 0.2 ml of a $2 \times 10^3$ µg/ml
aqueous solution was used.  Three mice were inoculated at each dose level
for each antibiotic.

The inoculation schedule and the results obtained are recorded in
Table XVIII.  In the doses used the antibiotics were not lethal for mice,
no deaths occurring over a 6-day observation period.

### Investigation of the toxicity of the supernatant from a broth culture
### of S. aureus 19.

In order to determine whether toxic products were involved,
S. aureus 19 was grown to an absorbance of 0.5 at 37°C and centrifuged
at 3000 rpm for 30 minutes in an IEC Model K-2 centrifuge, and the super-
natant removed.  Five mice were inoculated intraperitoneally with 0.2 ml
of the concentrated S. aureus 19 containing $8.8 \times 10^9$ cfu, and another 5

TABLE XVIII

LETHALITY OF ANTIBIOTICS FOR MICE

| Antibiotic | Dose (μg) | Death |
|---|---|---|
| Penicillin | 1000 | 0/3 |
| | 100 | 0/3 |
| Oxacillin | 2000 | 0/3 |
| | 200 | 0/3 |
| Vancomycin | 1000 | 0/3 |
| | 200 | 0/3 |
| Chloramphenicol | 1000 | 0/3 |
| | 400 | 0/3 |
| Bacitracin | 5000 | 0/3 |
| | 2000 | 0/3 |

mice, with 0.4 ml of the culture supernatant.

Observations of the animals after 24 hours showed no deaths among the animals receiving supernatant, and 100% deaths of animals receiving the concentrated cell suspension. The results thus show that the broth culture supernatant in a volume twice as large as that of S. aureus 19 suspensions used in this investigation was not lethal for mice, thus negating the possibility that the lethality of S. aureus 19 may be due to toxic products produced prior to inoculation.

### Additional controls

A. Heat inactivated Ø7.

In order to strengthen the argument that any protection observed in mice infected with S. aureus 19 and treated with Ø7 lysate was due to the presence of active Ø7, the following was adopted.

A portion (ca. 5 ml) of Ø7 lysate was inactivated by exposure in a temperature block at 70°C for 90 minutes. This inactivated suspension was then used to treat mice immediately after infection with S. aureus 19.

The results of this experiment showed that all 10 of the animals treated with the inactivated Ø7 suspension died within 24 hours while unheated lysate was able to afford 70% protection.

B. Nutrient Broth (NB $Ca^{++}$).

An experiment using sterile NB $Ca^{++}$ to treat mice infected with S. aureus 19 showed that the broth afforded no protection.

ACTION AND INTERACTION OF S. AUREUS 19,
∅7 AND ANTIBIOTICS IN MICE.

In vivo experiments were undertaken with S. aureus 19 to determine
the extent of the interaction of this bacterial strain, ∅7, and anti-
biotics as revealed by changes in the mortality of infected animals.

Response of mice inoculated with S. aureus 19 to different doses of
antibiotics

The experiment to be described was conducted in order to determine
what dose level of chloramphenicol, vancomycin, penicillin, and oxacillin
were effective in protecting mice which had been inoculated intraperi-
toneally with S. aureus 19.

At each dose level of antibiotic 3 mice were inoculated intraperi-
toneally with 0.2 ml of concentrated S. aureus 19 suspension containing
$1.3 \times 10^9$ cfu.  Immediately thereafter, antibiotics in the doses
indicated in Table XIX were also introduced intraperitoneally.  The anti-
biotics were contained in 0.2 ml of an aqueous solution except in the
case of chloramphenicol, where volumes ranging from 0.2 - 0.5 ml were
employed.

The results, listed in Table XIX, show that 1000 µg of penicillin
were able to protect one of the three mice infected with S. aureus 19;
Oxacillin in a 2000 µg dose protected two of three mice, and 1000 µg
vancomycin protected three of three.  Observations on the mice were
recorded after 24 hours, and the results did not change over a 3-day period.

TABLE XIX

RESPONSE OF MICE INFECTED WITH S. AUREUS 19
TO DIFFERENT DOSES OF ANTIBIOTICS.

|  |  | Observations (1 - 3 days) | |
| --- | --- | --- | --- |
| Antibiotic | Dose (µg) | Dead | Alive |
| Chloramphenicol | 1000 | 2 | 1 |
|  | 800 | 3 | 0 |
|  | 600 | 3 | 0 |
|  | 400 | 3 | 0 |
| Penicillin | 1000 | 2 | 1 |
|  | 100 | 3 | 0 |
|  | 10 | 3 | 0 |
| Oxacillin | 2000 | 1 | 2 |
|  | 200 | 1 | 2 |
|  | 20 | 3 | 0 |
| Vancomycin | 1000 | 0 | 3 |
|  | 200 | 1 | 2 |
|  | 50 | 0 | 3 |

Infecting dose of S. aureus 19   $1.3 \times 10^9$ cfu

The influence of Ø7 and each of the antibiotics chloramphenicol, vancomycin, penicillin, and oxacillin on the mortality of mice inoculated with S. aureus 19.

A number of experiments were performed to determine the extent to which the interaction of S. aureus 19, Ø7, and chloramphenicol, vancomycin, penicillin, and oxacillin affected the lethality of intra-peritoneally inoculated S. aureus 19 for mice.

Two dose levels of each antibiotic and two dose levels of Ø7 were examined using the standard inoculum of S. aureus 19. The Ø:cell ratios studied were, for "low dose phage" ca. 3:1, and for "high dose phage" ca. 11:1. The antibiotic doses were as follows:

|  | Low dose (µg) | High dose (µg) |
|---|---|---|
| Chloramphenicol | 200 | 1000 |
| Vancomycin | 50 | 200 |
| Penicillin | 10 | 1000 |
| Oxacillin | 20 | 2000 |

Each experiment included appropriate controls in which mice were infected with S. aureus 19 and no other treatment followed. Additional controls consisted of mice infected with S. aureus 19 and then treated with both high and low doses of Ø7, and high and low doses of each antibiotic.

Each experimental unit consisted of a randomized set of 10 mice. Using a 2.5 ml hypodermic syringe and 26G ½ inch needle, a 0.2 ml inoculum of concentrated log phase suspension of S. aureus 19 containing

ca. $8.8 \times 10^8$ cfu was injected intraperitoneally into the right side of each animal. When Ø7 treatment, antibiotic treatment, or combined Ø7 and antibiotic treatment was required, it was administered intraperitoneally into the left side immediately after injection of the bacteria. Both the Ø7 dose and antibiotic dose were administered in 0.2 ml volume. For combined antibiotic and Ø7 treatment, the two agents were mixed together and also given in a 0.2 ml volume, except in the case of chloramphenicol, where 0.2 ml of Ø7 and antibiotic were injected separately, immediately after inoculation of each mouse with S. aureus 19.

The animals were observed for a 24-hour period and the results are presented in Tables XX, XXI, XXII, and XXIII under appropriate headings.

From the values of each of the Tables XX - XXII, the following interactions were tested for significance using the $\chi 2$ statistic at the 95% level.

1. Infected mice vs Ø-treated mice.

2. Infected mice vs antibiotic-treated mice.

3. Infected mice vs antibiotic and Ø-treated mice.

4. Ø-treated mice vs antibiotic and Ø treated mice.

5. Antibiotic-treated vs antibiotic and Ø-treated mice.

6. Ø-treated vs antibiotic-treated mice.

The $\chi 2$ values corresponding to the above interactions for each of Tables XX - XXIII are presented in Table XXIV.

TABLE XX

INTERACTION OF S. AUREUS 19, LOW-DOSE Ø7

AND LOW-DOSE ANTIBIOTIC IN MICE

| Antibiotic | Dose (μg) |
|---|---|
| Chloramphenicol | 200 |
| Vancomycin | 50 |
| Penicillin | 10 |
| Oxacillin | 20 |

| Treatment of Mice | 24-hour observation | |
|---|---|---|
| | Dead | Alive |
| 1. S. aureus 19 | 9 | 1 |
| 2. S. aureus 19 + Ø7 | 4 | 6 |
| 3. S. aureus 19 + chloramph. | 10 | 0 |
| 4. S. aureus 19 + chloramph. + Ø7 | 6 | 4 |
| 5. S. aureus 19 + van. | 5 | 5 |
| 6. S. aureus 19 + van. + Ø7 | 2 | 8 |
| 7. S. aureus 19 + pen. | 10 | 0 |
| 8. S. aureus 19 + pen. + Ø7 | 4 | 6 |
| 9. S. aureus 19 + oxac. | 9 | 1 |
| 10. S. aureus 19 + oxac. + Ø7 | 8 | 2 |

Infecting dose of S. aureus 19 was ca. $8.8 \times 10^8$ cfu

Ø:cell ratio, ca. 3:1

TABLE XXI

INTERACTION OF S. AUREUS 19, HIGH-DOSE Ø7

AND LOW-DOSE ANTIBIOTIC IN MICE.

| Antibiotic | Dose ($\mu$g) |
|---|---|
| Chloramphenicol | 200 |
| Vancomycin | 50 |
| Penicillin | 10 |
| Oxacillin | 20 |

| Treatment of mice | 24-hour observation | |
|---|---|---|
| | Dead | Alive |
| 1.  S. aureus 19 | 9 | 1 |
| 2.  S. aureus 19 + Ø7 | 2 | 8 |
| 3.  S. aureus 19 + chloramph. | 10 | 0 |
| 4.  S. aureus 19 + chloramph. + Ø7 | 4 | 6 |
| 5.  S. aureus 19 + van. | 5 | 5 |
| 6.  S. aureus 19 + van. + Ø7 | 2 | 8 |
| 7.  S. aureus 19 + pen. | 9 | 1 |
| 8.  S. aureus 19 + pen. + Ø7 | 4 | 6 |
| 9.  S. aureus 19 + oxac. | 8 | 2 |
| 10. S. aureus 19 + oxac. + Ø7 | 0 | 10 |

Infecting dose of S. aureus 19 was ca 8.8 x $10^8$ cfu

Ø:cell ratio, ca. 11:1

TABLE XXII

INTERACTION OF S. AUREUS 19, LOW DOSE Ø7

AND HIGH DOSE ANTIBIOTIC IN MICE.

| Antibiotics | Dose (µg) |
|---|---|
| Chloramphenicol | 1000 |
| Vancomycin | 200 |
| Penicillin | 1000 |
| Oxacillin | 2000 |

| | Treatment of mice | 24-hour observation | |
|---|---|---|---|
| | | Dead | Alive |
| 1. | S. aureus 19 | 9 | 1 |
| 2. | S. aureus 19 + Ø7 | 4 | 6 |
| 3. | S. aureus 19 + chloramph. | 7 | 3 |
| 4. | S. aureus 19 + chloramph. + Ø7 | 2 | 8 |
| 5. | S. aureus 19 + van. | 2 | 8 |
| 6. | S. aureus 19 + van. + Ø7 | 1 | 9 |
| 7. | S. aureus 19 + pen. | 5 | 5 |
| 8. | S. aureus 19 + pen. + Ø7 | 2 | 8 |
| 9. | S. aureus 19 + oxac. | 2 | 8 |
| 10. | S. aureus 19 + oxac. + Ø7 | 1 | 9 |

Infecting dose of S. aureus 19 was ca. $8.8 \times 10^8$ cfu

Ø:cell ratio, ca. 3:1

TABLE XXIII

INTERACTION OF S. AUREUS 19, HIGH DOSE Ø7

AND HIGH DOSE ANTIBIOTIC IN MICE

| Antibiotics | Dose (μg) |
|---|---|
| Chloramphenicol | 1000 |
| Vancomycin | 200 |
| Penicillin | 1000 |
| Oxacillin | 2000 |

| Treatment of mice | 24-hour observation | |
|---|---|---|
| | Dead | Alive |
| 1.  S. aureus 19 | 9 | 1 |
| 2.  S. aureus 19 + Ø7 | 3 | 7 |
| 3.  S. aureus 19 + chloramph. | 4 | 6 |
| 4.  S. aureus 19 + chloramph. + Ø7 | 2 | 8 |
| 5.  S. aureus 19 + van. | 3 | 7 |
| 6.  S. aureus 19 + van. + Ø7 | 2 | 8 |
| 7.  S. aureus 19 + pen. | 7 | 3 |
| 8.  S. aureus 19 + pen. + Ø7 | 2 | 8 |
| 9.  S. aureus 19 + oxac. | 2 | 8 |
| 10.  S. aureus 19 + oxac. + Ø7 | 3 | 7 |

Infecting dose of S. aureus 19, ca. $8.8 \times 10^8$ cfu.

Ø:cell ratio, ca. 11:1

## TABLE XXIV

## STATISTICAL ANALYSIS OF TABLES XX – XXIII

| Interaction | | Table XX | Table XXI | Table XXII | Table XXIII |
|---|---|---|---|---|---|
| Adjusted 2 | | | | | |
| 1. Infected vs Ø-treated mice | | $3.52^{ns}$ | 7.03** | $3.52^{ns}$ | 5.20 * |
| 2. Infected vs antibiotic-treated | Chloramph. | 0.00 | $0.00^{ns}$ | $0.31^{ns}$ | 3.52 |
| | Van. | $2.14^{ns}$ | $2.14^{ns}$ | 7.27* | 5.20* |
| | Pen. | $0.00^{ns}$ | $0.00^{ns}$ | $2.14^{ns}$ | $0.31^{ns}$ |
| | Oxac. | $0.00^{ns}$ | $0.00^{ns}$ | 7.27** | 7.27** |
| 3. Infected vs antibiotic + Ø-treated | Chloramph. | $1.07^{ns}$ | $3.52^{ns}$ | 7.27** | 7.27** |
| | Van. | 7.27** | 7.27** | 9.80** | 7.27** |
| | Pen. | $1.06^{ns}$ | $3.52^{ns}$ | 8.18** | 7.27** |
| | Oxac. | $0.00^{ns}$ | 12.93** | 9.80** | 5.20* |
| 4. Ø-treated vs antibiotic-Ø-treated | Chloramph. | $0.20^{ns}$ | $2.24^{ns}$ | $0.24^{ns}$ | $0.00^{ns}$ |
| | Van. | $0.24^{ns}$ | $0.00^{ns}$ | $1.07^{ns}$ | $0.00^{ns}$ |
| | Pen. | $0.00^{ns}$ | $0.24^{ns}$ | $0.24^{ns}$ | $0.00^{ns}$ |
| | Oxac. | $1.88^{ns}$ | $0.56^{ns}$ | $1.07^{ns}$ | $0.00^{ns}$ |
| 5. Antibiotic-treated vs antibiotic-Ø-treated | Chloramph. | $2.81^{ns}$ | 5.95* | $3.23^{ns}$ | $0.24^{ns}$ |
| | Van. | $0.88^{ns}$ | $0.88^{ns}$ | $0.00^{ns}$ | $0.00^{ns}$ |
| | Pen. | 5.95* | 4.06* | $1.06^{ns}$ | $3.23^{ns}$ |
| | Oxac. | $0.00^{ns}$ | 10.21** | $0.00^{ns}$ | $0.00^{ns}$ |
| 6. Ø-treated vs antibiotic-treated | Chloramph. | 5.95* | 10.21** | $0.81^{ns}$ | $0.00^{ns}$ |
| | Van. | $0.00^{ns}$ | $0.88^{ns}$ | $0.24^{ns}$ | $0.00^{ns}$ |
| | Pen. | 5.95* | 7.27** | $0.00^{ns}$ | $1.80^{ns}$ |
| | Oxac. | $3.52^{ns}$ | 5.00* | $0.24^{ns}$ | $0.00^{ns}$ |

Ø = Ø7  
ns = not significant  
* = significant  
** = highly significant

The $\chi 2$ values for the interaction of $\emptyset7$ treated and antibiotic $\emptyset7$ treated mice from each of Tables XX – XXIII show that in no instance did the samples indicate a difference greater than could be attributed to chance.  This leads to the conclusion that for the doses of antibiotic and $\emptyset7$ used in these experiments to treat mice inoculated intraperitoneally with S. aureus 19, the results do not allow the interpretation that bacteriophage and antibiotic act additively or synergistically.

RATE OF DEATH OF MICE INOCULATED WITH S.AUREUS 19

AND THE INFLUENCE OF Ø7, ANTIBIOTICS AND

STAPHYLOCOCCAL ANTITOXIN.

Reports of reduced survival following delay in administration of

bacteriophage to mice which had received a lethal intraperitoneal

inoculum of S. aureus have been made by Bartell and co-workers (7, 8).

Other studies seem to suggest that death within 24 hours in mice

infected with S. aureus was due to toxic components and that the final

outcome was determined at an early stage.  Accordingly it was decided

to examine some of these parameters as well as the extent to which

chloramphenicol, vancomycin, penicillin, and oxacillin affect the time

when infected mice died.

S. aureus 19, Ø7, penicillin interaction  (time of death)

The first experiment in this series was performed using male

mice (ca. 25 gm) 400 μg of penicillin, a standard inoculum of S. aureus 19

and Ø7 suspension yielding a Ø:cell ratio of ca. 3:1.  The method of

inoculation was the same as above.

The results showed that mice receiving the standard S. aureus 19

died early and at a rapid rate.  The first death was recorded 2½ hours

post-infection and by 6 hours 9 of the 10 infected mice had died.  The

mice treated with Ø7 showed two deaths in 3 hours; no further deaths

occurred within 3 days.  Penicillin-treated mice showed a delay in the

onset of death, first deaths occurring 4 hours post-infection and

proceeding at a slower rate until by 9 hours nine of the infected mice

had died. Thus, although 400 µg of penicillin was able to prolong the

survival of the mice for a few hours the number dead in 24 hours was

unaltered. Combined penicillin and Ø7 treatment effected 100% survival

of the animals. When the administration of Ø7 was delayed for 15 minutes,

the onset and rate of death were delayed, but the number which died in 24

hours was only one less than the untreated controls.

The results of this experiment prompted further investigations

which are reported below. Because of the lack of a sufficient number of

male mice, females were used for the most part in these latter experiments.

The responses of male and female mice to intraperitoneally inoculated

S. aureus 19 and to treatment with Ø7 immediately following infection

were compared, and served as controls for the other experimental units.

Comparison of the responses of male and female mice to intraperitoneal

inoculation with S. aureus 19, and to immediate treatment with Ø7.

The method of inoculation and standard dose of S. aureus 19 was

the same as before. The Ø:cell ratio used was ca. 10:1.

The results recorded in Table XXV (a) show 4 female and 6 male

mice dead by 3½ hours, with 9 female mice dying in 4 hours and 9 males in

4½ hours. The final figures for 24 hours are 9 female and 10 male mice

dead. The male series showed and early death which could probably be

attributed to shock. The course of events using the male and female

animals would therefore appear to be similar.

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Rate of death in mice as influenced by the interaction of S. aureus 19, Ø7, and each of the antibiotics, chloramphenicol, vancomycin, penicillin, and oxacillin.

The Ø:cell ratio used was ca. 10:1, with the standard inoculum of S. aureus 19 of ca.8.8 x $10^8$ cfu. The antibiotic doses employed were: chlorampenicol - 600 µg, penicillin - 400 µg, and oxacillin - 1000 µg. Results are recorded in Table XXVI.

For the antibiotics chloramphenicol, penicillin, vancomycin and oxacillin, there was a reduction in the death rate. It can also be seen (Table XXVI) in the case of chloramphenicol and penicillin that a 15-minute delay in the administration of Ø7 resulted in considerable loss of protection. Comparable observations for the vancomycin and oxacillin series were obscured by the high level of protection which these anti-biotics by themselves afforded.

The effect of S. aureus 19 on antitoxin pre-treated mice.

In this experiment 10 mice were given intraperitoneally 500 units of staphylococcal antitoxin (Connaught, Canada), ca. 2½ hours before intraperitoneal challenge with S. aureus 19.

The results recorded in Table XXV (b) show that the onset of death was considerably delayed, since none of the mice died before 9 hours post-infection. However, by 15 hours, 7 animals died and this was increased to 8 at 24 hours.

TABLE XXV (a)

COMPARISON OF THE RESPONSE OF MALE AND FEMALE MICE TO INTRAPERITONEAL

INFECTION WITH S. AUREUS 19 AND TO IMMEDIATE TREATMENT WITH Ø7

Post-infection survivors

| Treatment | Hours | | | | | | | | | | | | | | | | Days | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 2½ | 3 | 3½ | 4 | 4½ | 5 | 5½ | 6 | 6½ | 7 | 7½ | 8 | 8½ | 9 | 9½ | 1 | 2 | 3 |
| ♀ mice + S. aureus 19 | 10 | 10 | 10 | 4 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| ♂ mice + S.aureus 19 | 9 | 9 | 9 | 6 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | | 0 | 0 | 0 |
| ♀ mice + S. aureus 19 + Ø7 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | | 10 | 10 | 10 |
| ♂ mice + S.aureus 19 + Ø7 | 10 | 10 | 10 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | | 9 | 9 | 9 |

Phage:cell ratio, ca. 10:1

TABLE XXV (b)

THE EFFECT OF S. AUREUS 19 ON MICE PRE-TREATED WITH STAPHYLOCOCCAL ANTITOXIN

Post-infection survivors

| | Hours | | | | | | | | | | | | | | | | Days | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 2½ | 3 | 3½ | 4 | 4½ | 5 | 5½ | 6 | 6½ | 7 | 7½ | 8 | 8½ | 9 | 15 | 1 | 2 | 3 |
| Anti-alphatoxin pre-treated mice + S. aureus 19 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 3 | 2 | 1 | 1 |

TABLE XXVI

RATE OF DEATH IN MICE AS INFLUENCED BY THE INTERACTION OF S.AUREUS 19

Ø7 AND EACH OF THE ANTIBIOTICS CHLORAMPHENICOL, VANCOMYCIN,

PENICILLIN, AND OXACILLIN

Post-infection survivors

| Treatment | Hours | | | | | | | | | | | | | | | Days | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 2½ | 3 | 3½ | 4 | 4½ | 5 | 5½ | 6 | 6½ | 7 | 7½ | 8 | 8½ | 9 | 1 | 2 | 3 |
| Chloramph., 600 µg | 10 | 10 | 10 | 10 | 9 | 7 | 6 | 4 | 3 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Chloramph. + Ø7 | 10 | 10 | 10 | 10 | 10 | 10 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 8 | 8 | 8 |
| Chloramph. + Ø7, 15 min. later | 10 | 10 | 10 | 10 | 10 | 10 | 7 | 5 | 5 | 5 | 4 | 4 | 3 | 3 | 3 | 3 | 3 | 3 |
| Van., 100 µg | 10 | 10 | 10 | 10 | 10 | 10 | 9 | 9 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 |
| Van. + Ø7 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Van. + Ø7, 15 min. later | 10 | 10 | 10 | 10 | 10 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 |
| Pen., 400 µg | 10 | 10 | 10 | 8 | 6 | 4 | 3 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 |
| Pen. + Ø7 | 10 | 10 | 10 | 10 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 |
| Pen. + Ø7, 15 min. later | 10 | 10 | 10 | 10 | 10 | 8 | 7 | 5 | 4 | 3 | 2 | 2 | 2 | 2 | 1 | 1 | 1 | 1 |
| Oxac., 1000 µg | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 9 | 9 | 9 | 8 | 8 | 8 |
| Oxac. + Ø7 | 10 | 10 | 10 | 10 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 |
| Oxac. + Ø7, 15 min. later | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 9 | 9 | 9 |

Ø:cell ratio, 10:1

DISCUSSION OF IN VIVO INVESTIGATIONS.

S. aureus PS7 given intraperitoneally to 20 - 30 gm mice was found
to be relatively innocuous.  An inoculum in the order of $10^{10}$ cfu was
required to kill the animals.  This dose was far in excess of that
(1 - 2 x $10^9$ cfu) for S. aureus phage type 80/81 found by Muir and
Blakemore (60) and by Bartell et al. (7) to kill more than 80% of mice.

Prompted by an early observation of Van de Velde (93), viz. that
in vivo passage of an organism may increase its virulence, S. aureus PS7
was subjected to successive intraperitoneal transfer in mice.  No
augmentation of virulence of this staphylococcal strain for mice by the
intraperitoneal route was obtained.  In the light of this, a number of
other staphylococcal isolates were examined for their lethality to mice
and of these, S. aureus strain 19 was selected for further study, since
it had an $LD_{50}$ of 2.9 x $10^7$ cfu and possessed an antibiotic sensitivity
spectrum similar to that of S. aureus PS7.  In addition strain 19 was
able to act as host for Ø7 with an EOP of 83% relative to strain PS7.

An infecting dose (8.8 x $10^8$ cfu) of strain 19 given intraperi-
toneally was found to kill 9 of 10 mice in 4 - 6 hours.  This result is
comparable to the observations of Smith et al. (85) who obtained 8 - 9
deaths from groups of 10 mice intraperitoneally infected with $10^{7-8}$ cfu
of S. aureus (Smith and Dubos 1956) and of Muir and Blakemore (60) who
obtained 96% death in 5 - 6 hours when mice infected with

$1 - 2 \times 10^9$ cfu of a strain of S. aureus of phage type 80/81.

Many staphylococcal products have been correlated with virulence, but despite the implication of some of these in disease production, there is as yet no evidence to pinpoint the precise mechanism involved in producing death in experimentally infected animals, or in associating it with any single biochemical event. The delay in the onset of death which was observed when mice were pre-treated with staphylococcus antitoxin is in agreement with the observations of Kapral (33) and of Mathieu et al. (56). Kapral (33) has also obtained death in mice by the administration of purified alpha-toxin and has demonstrated that mutants of S. aureus unable to produce alpha toxin in vitro showed marked reduction in lethality for mice. Moreover he has demonstrated that an amount of toxin equivalent to the $LD_{50}$ for pre-formed alpha-toxin could be produced in 3 - 6 hours by the initial dose of cocci which were lethal to mice, and that for the production of alpha-toxin, bacteria must be actively dividing. This latter observation is at variance with the reported results of Mathieu et al. (56) who, using a thymineless mutant of S. aureus Wood 46 which did not multiply in the peritoneal cavity, were still able to obtain delayed death in mice by pre-treatment with anti-toxin. While these observations suggest that toxins, and in particular alpha toxin, are important factors in the lethality of S. aureus they do not in themselves provide an entirely satisfactory explanation of the mechanisms by which death is inflicted.

Penicillin, oxacillin, vancomycin, and chloramphenicol in the

doses used in this investigation were not lethal for mice.  Usually in

the assessment of drug toxicity many other parameters are examined, e.g.

the effect on erythropoetic,kidney,and liver functions as well as on

other biological phenomena.  In the present investigation however, quick

deaths within 24 hours were of primary concern.

As was expected, the degree of protection in mice intraperito-

neally infected with strain 19 improved with increasing doses of anti-

biotic.  An observation worthy of comment is the high level of protection

afforded by vancomycin as compared with penicillin, despite the much

greater effectiveness of the latter antibiotic in vitro.  A possible

explanation for this may lie in the greater binding and hence reduced

effectiveness of penicillin by proteins in vivo.  The relative insolu-

bility of chloramphenicol in aqueous solution, as well as its rapid

inactivation and excretion, as shown by Glazko et al. (24), may provide

an explanation for its relative ineffectiveness in the doses used.

Although the $\chi 2$ test for the interaction of $\emptyset$-treated and

antibiotic + $\emptyset$-treated mice for each of Tables XX - XXIII show in no

instance a difference greater than could be attributed to chance, a

closer look at the experimental results, in particular those of Tables

XXII and XXIII, seem to suggest that there may indeed be some interaction

of $\emptyset$ and antibiotic leading to addition of protective action.  In Table

XXII the number of mice surviving after antibiotic plus $\emptyset 7$ treatment, was

for each experimental unit greater than for $\emptyset 7$ or for antibiotic alone.

Except for the case of oxacillin in Table XXIII, where there was one less

survivor for oxacillin plus Ø7, the number of survivors for Ø7 plus

antibiotic treatment was greater than for either antibiotic or Ø7 alone.

One is therefore inclined to the conclusion that while the results of

the experiments performed do not prove interaction of antibiotics and

Ø7 provide augmented protection in mice intraperitoneally infected with

S. aureus 19, some results nevertheless suggest that such an interaction

may be present but that the size of the individual samples may have been

too small to demonstrate this unequivocally.

There is ample evidence in the literature that bacteriophages

can traverse many anatomical barriers. Keller and Engley (38) have

shown that a Bacillus megaterium phage can pass through the gut and

even through the apparently intact skin of mice and be recovered from

the blood. Nungester and Watrous (65) have demonstrated the accumu-

lation of intravenously injected staphylococcal Ø in the spleen and

liver of rats. Keller and Engley (38) have also shown the presence of

intraperitoneally inoculated B. megaterium Ø in the blood, spleen,

liver, lungs, and brain of mice. Additional evidence for the in vivo

translocation of Ø particles is presented by Schultz and Neva (80),

who were able to find T2 coliphage in urine of intravenously inoculated

mice, and by Schultz and Frohlich (80) who found T5 coliphage in the

spinal fluid of dogs after intravenous injection. In addition, in vivo

interaction of Ø and bacteria has been conclusively demonstrated by

Bartell et al. (6)

When Ø7 is introduced intraperitoneally immediately after an

infecting dose of $\underline{S}$. $\underline{aureus}$ 19, it is readily apparent how the bacterial

inoculum could be reduced below its critical fatal level and result in

an increase in the number of surviving animals. What is not so

apparent, however, is why a delay of as little as 15 minutes should so

effectively abolish this protection. In the experiments where the

addition of Ø7 was delayed, a high level of protection was afforded

when 100 μg vancomycin and 1000 μg oxacillin were used, whereas a low

level of protection was afforded when 600 μg chloramphenicol and 400 μg

penicillin were used - where protection was high for antibiotic alone,

it was high for antibiotic plus delayed Ø7, and where low for anti-

biotic alone, it was also low for antibiotic plus Ø7. Viewed together

these observations suggest that 15 minutes after intraperitoneal

inoculation a sufficient number of staphylococci are isolated in such

a manner that they are still accessible to diffusing antibiotic but

inaccessible to Ø7. The most likely explanation is suggested by the

observations of Kapral (34) and other workers, that after intraperi-

toneal inoculation, coagulase-positive staphylococci quickly form

clumps due to the reaction of bound coagulase and fibrinogen in the

peritoneal fluid. The clumps become surrounded by leucocytes already

present in the peritoneal cavity. This clumping phenomenon, as well

as the mechanical obstruction provided by fibrin formation and

adhering leucocytes, may serve to reduce effective interaction of

bacteria with Ø7 administered later so that the infecting inoculum is

not diminished below its critical level, as would otherwise occur if

both agents were administered simultaneously.  Bacteria, however,

apparently remain accessible to diffusible antibiotic molecules.

IV

SUMMARY AND CONCLUSION

# SUMMARY AND CONCLUSION

The interaction of the antibiotics penicillin, D-cycloserine, vancomycin, bacitracin, and chloramphenicol with Ø7 and S. aureus PS7 was investigated in vitro. A complementary in vivo investigation was conducted using Ø7, S. aureus strain 19 and the antibiotics penicillin, oxacillin, chloramphenicol and bacitracin.

## Summary

### In vitro

In vitro investigation led to the following findings.

(1)     The growth of Ø7 with S. aureus PS7 in $Ca^{++}$ supplemented nutrient broth at $37^{o}C$ resulted in a burst size of ca. 95;  120 minutes after infection there was a 140-fold increase in titrable Ø.

(2)     Penicillin, D-cycloserine, and vancomycin in concentrations of 10 µg/ml, 100 µg/ml, and 100 µg/ml respectively caused a considerable reduction in Ø yield, the amount of titrable Ø7 120 minutes after infection being 2, 3, and 5 times the input.  With 100 µg/ml bacitracin and 20 µg/ml chloramphenicol the titrable Ø7 120 minutes after infection was only ½ the input.

(3)     The adsorption of Ø7 to S. aureus PS7 was rapid and ca. 95% completed in 5 minutes.

(4)     The adsorption of S. aureus PS7 was not affected by the presence of penicillin, D-cycloserine, bacitracin, vancomycin, chloramphenicol or

oxacillin added 1 minute before Ø7 and present during the adsorption period.

(5)    The addition of penicillin and D-cycloserine in growth-inhibiting concentrations to Ø-infected S. aureus PS7 suspensions, together with, or shortly after, Ø infection, did not inhibit rapid lysis of the bacterial suspensions.  On the contrary, lysis was speeded up.

(6)    Bacitracin and vancomycin added together with Ø7 caused inhibition of rapid lysis of S. aureus PS7, the effect of vancomycin being more pronounced.

(7)    Pre-treatment of S. aureus PS7 with penicillin, D-cycloserine, vancomycin and bacitracin caused inhibition of rapid lysis of the staphylococci by Ø7.  This effect was greatest for vancomycin and least for penicillin, and was more evident the longer the interval between addition of antibiotic and the later addition of Ø7.

(8)    In contrast to the different degrees of lysis of S. aureus PS7 by Ø7 in the presence of penicillin, D-cycloserine, vancomycin and bacitracin, chloramphenicol caused virtually complete inhibition of lysis when added before Ø7, together with Ø7, or up to 30 minutes after Ø7.

    In vivo.

(1)    S. aureus PS7 was not lethal for mice by the intraperitoneal route, and increased virulence was not obtained as a result of successive intra-peritoneal transfers.

(2)    The $LD_{50}$ for log-phase S. aureus strain 19 for 20 - 30 gm mice by the intraperitoneal route was $2.9 \times 10^7$ cfu.

(3)  Male and female mice responded similarly to intraperitoneal inoculation with S. aureus 19.

(4)  Intraperitoneal inoculation of Ø7 immediately after intraperitoneal inoculation of S. aureus 19 was effective in increasing the number of animals which survived.

(5)  Witholding Ø7 treatment for 15 minutes abolished its protective action for mice previously inoculated intraperitoneally with S. aureus 19.

(6)  Pre-treatment of mice with 500 units of antistaphylococcal antitoxin caused a delay in the onset of death in mice subsequently infected with S. aureus 19 but did not significantly influence the final number which died.

(7)  Statistical analysis of the results did not reveal any obvious interaction of Ø7 and antibiotics where animal survival was concerned. Some results did suggest that such an interaction may have been present although it was obscured because of the small size of the sample units used in these experiments.

CONCLUSION

The question whether ∅ and antibiotic interact to produce any augmentation of antibacterial activity is exceedingly complex. It is little wonder therefore that the problem has so far been attacked only in broad general terms and that as a result very little definitive information has been obtained. Even in those instances where attempts have been made to provide an answer based on empirical observation of the lysis of bacterial suspensions in vitro, or of the mortality rate of infected animals, the results have proved difficult to interpret.

The present investigation confirms the observations of previous workers such as Krueger (43) and Elford (18) that penicillin-treated bacterial suspensions could be made to lose their turbidity in the presence of ∅ earlier than would have been the case with either agent alone. This investigation also shows that similar results were obtained for D-cycloserine, and for vancomycin and bacitracin if these latter antibiotics were added at appropriate intervals after ∅7. Viewed in broad general terms, therefore, there is no hesitation in concluding that antibiotic and ∅ can be made to affect bacterial suspensions additively.

Penicillin, D-cycloserine, vancomycin and bacitracin have both growth-inhibiting and lytic properties, as does ∅7. There is, however, overwhelming support for the contention that antibiotics are more likely to inhibit than to augment ∅ activity. For these four antibiotics which affect muerin synthesis, it may be expected that some weakening of the

cell wall structure would occur, and the contribution of this to

facilitating lysis by ∅7 would be expected  to be influenced by any

adverse effects on ∅ activity which the antibiotics may possess.  Where

additive action of ∅ and antibiotic was demonstrated it seems likely

that the major portion of this lytic activity was associated with the ∅,

the antibiotic acting mainly to prevent further bacterial growth so that

there were fewer bacteria to lyse and hence earlier clearing of

bacterial suspensions was observed.

The in vivo investigations in mice did not reveal significant

interaction of ∅7 and antibiotics leading to additive protection from

intraperitoneal infection with S. aureus strain 19.  The results do

however suggest that if a sufficiently large number of animals were

tested, such an interaction may be revealed.

In conclusion, it would be remiss not to reiterate the generally

accepted but often ignored truism that conclusions in regard to the anti-

bacterial activity of antimicrobial agents in vitro, while giving some

indication of the responses which may be expected, cannot be unreservedly

transposed to the in vivo situation.  In particular, the numerous

humoral and cellular immunological mechanisms which are known to be

involved in the defence of an infected animal must always be considered.

Their evaluation is however made more difficult by the fact that experi-

mentally they can neither be readily included in vitro, nor excluded in

vivo.

V

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VI

APPENDIX

Appendix 1

<u>Antibiotics Used and Their Source</u>

1.      Penicillin G. (Potassium)

        Ayerst Laboratories, Montreal, Canada.

2.      Seromycin (D-cycloserine)

        Eli Lilly and Co., Indianapolis, U.S.A.

3.      Vancomycin (Sulfate)

        Potency standard 962 units/mg

        Eli Lilly and Co., Indianopolis, U.S.A.

4.      Bacitracin

        Secondary: Ref. Standard

        Eli Lilly and Co., Indianapolis, U.S.A.

5.      Chloramphenicol

        Parke, Davis and Co. Ltd.

        Montreal, Canada.

6.      Oxacillin (Sodium)

        Potency, 900 $\mu$g/mg

        Bristol Laboratories, Syracuse, N.Y., U.S.A.